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IMMUNOLOGY AS A TOOL IN BIOLOGICAL RESEARCH[1]

IMMUNOCHEMICAL APPROACHES TO BIOLOGICAL PROBLEMS

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I VENTURE to address this gathering of geneticists and zoologists with an exhilaration engendered by a sense of the daring involved in an excursion into well-explored fields of knowledge remote from those into which my own work has extended. I trust, however, you will forgive this excursion or incursion, as it is intended more to remind you of progress already made in your fields along immunochemical lines, rather than to suggest the adoption of wholly foreign techniques and ideas.

However, before reviewing these applications, it might be well to describe again in modern chemical terms some of the concepts fundamental to immunology.

Knowledge of antigens, or the substances stimulating immune responses in animals, has been greatly extended in recent years. Thanks to chemical fractionations, the ultracentrifuge, the Tiselius electrophoresis apparatus and other powerful tools, one may no longer consider horse serum, for example, or an animal or bacterial cell, as "an antigen," but must recognize it as a collection of antigens,

[1] Four papers from a symposium scheduled to be presented by the Genetics Society of America at the annual meeting of the American Association for the Advancement of Science, which was cancelled at the request of the Office of Defense Transportation, December, 1942.

each with distinct properties and potencies. Many immunological observations were and are difficult to interpret because this complexity was not taken into account. It is also apparent that many antigens are proteins and that most proteins are antigenic. Much work has been done showing that denaturation as well as introduction of the most varied chemical groupings at almost any point of substitution results in a definite change in immunological specificity.

Now most of you will remember that some time ago, in Avery's laboratory, we found that type specificity among the encapsulated bacteria depended upon another kind of antigen. This type specificity was due to a peculiar group of polysaccharides resistant to the usual sugar-splitting enzymes. The specific polysaccharide of each pneumococcus type, for example, was different from those of other types, and could be characterized by its distinctive physical and chemical properties. The sugars from types II and III pneumococcus were obtained free from nitrogen, and were the first instances in which immune specificity had been rigorously demonstrated in a class of substances other than proteins.

With this brief discussion of specificity as a basis, what can be said about the requisite conditions for antigenicity? It is obvious that we must have a complex structure and large molecules, and one of the important things seems to be the repetition of structural units. This is a highly probable consequence of the modern views of protein structure. We also know that the specific carbohydrate of type III pneumococcus, for instance, is made up of many cellobiuronic acid units. Some multiple of this unit must function as the immunologically reactive grouping, for when the carbohydrate is partially broken down by mild hydrolysis the fragments of two or more units still react in anti-pneumococcus type III horse serum. Therefore we may assume that in order to function fully as an antigen a substance of large molecular size must be of such nature as to allow repetition of cer-

tain structural units. Possibly for this reason ordinary lipids do not appear to have a clear-cut antigenic function.

I think Dr. Landsteiner would add that any simple chemical substance may also function as an antigen especially if the chemical properties are such as to allow its combination with protein to form new antigens. Complex structure is not necessary, therefore, if a number of molecules of a smaller entity can combine to form part of a larger structure.

With regard to antibodies, the immune substances engendered in animals as a result of the antigenic stimulus, we are in a position to be equally definite. Use of new quantitative chemical microanalytical methods made it possible to measure antibodies in sera in actual weight units. One could, for the first time, express antibodies in terms of specific nitrogen per cubic centimeter of serum, because after precipitation with a slight excess of antigen non-specific material could be washed out. Since the amount of nitrogen in the added antigen is known this may be subtracted and the residual nitrogen in the washed precipitate is due to the antibodies. Highly purified antibody solutions obtained as a consequence of information gained by these new methods were examined in the ultracentrifuge and electrophoresis apparatus and were shown to have the properties of typical serum proteins.

Buchner's hypothesis that antibody contained fragments of antigen was proposed at a time when the actual nature of antibodies was not understood. This hypothesis never appealed to the chemist because in a number of instances like repels like, rather than attracts. In 1932 Breinl and Haurowitz proposed a theory that antibodies are formed by a modification of the normal process of serum globulin synthesis as a result of penetration of antigen or specific portions of the antigen to the site of globulin synthesis. The disturbance so brought about influences the course of that synthesis in a sense characteristic of the antigen so that when the modified globulin appears in the circulation and again encounters the antigen

interaction is possible. This not very clear picture was later expressed in somewhat more definite form by Mudd. An extension of this hypothesis has recently been made by Pauling which is even more graphic and reasonable but as devoid of experimental basis as the Breinl and Haurowitz theory. The Pauling hypothesis carried a second idea—that if one could take normal globulin, denature it and fold it up again in the presence of antigen, artificial production of antibodies might be accomplished. Pauling now believes he has been successful in this, but such details of his experiments as have been published do not include complete controls. Burnet has recently proposed the origin of antibodies through modification by antigen of intracellular proteases which provide the framework for synthesis of partial replicas of themselves (globulins or antibodies). This would provide for antibody formation after destruction of antigen and for progressive changes in antibodies with successive immunizations.

These theories of antibody formation have been given a physiological basis in recent years by Dr. Florence Sabin as a result of experimental work with a red protein dye. Dr. Sabin has observed macrophages in the omentum and cells of the reticulo-endothelial system and found that, at a certain stage of development, surface layers which form folds waving back and forth finally disappeared as if they were being extruded from these cells. She believes this to be the source of serum globulins and that the presence of an antigen (for example, the red protein dye) results in the specific modification of these globulins into the appropriate antibody.

Now for a few applications to genetics and biology:

Nuttall's pioneer work on the mapping of biological relationships through the study of the interaction of animal sera with antibodies formed when these sera are injected into a standard animal such as the rabbit was most fruitful and has been extended by numerous workers. The immunochemist has shown that interpretation of the com-

plex findings is often simplified if a single protein is used, rather than serum, which we know to be a complex mixture of albumin, at least three globulins, complement with its four components, and other minor substances, all or most of which may function as antigens and cause overlapping or zone effects in reactions with antisera. Nor is it certain that precipitation in different antisera is always due to the same antigen when such a mixture is used.

An extreme instance of the simplification wrought by the use of pure, crystalline proteins was the demonstration by Landsteiner and myself of the non-identity or identity of the oxyhemoglobins of various species by a physical-chemical (solubility) method as well as by the serological technique. By use of the quantitative precipitin method, in which the amount of antibody nitrogen precipitated by a single purified antigen is measured, information as to species relationships may be gained that is unobtainable by qualitative measurements. In this way Stokinger and I were able to show the close relationship, but lack of identity, of sheep and bovine thyroglobulins, and to demonstrate that even this organ-specific globulin hormone possessed a species-specificity entirely independent of that of the corresponding serum globulins. With the same quantitative method Treffers, Moore and I were able to give a plausible explanation for the differences shown by normal horse γ-globulin and antipneumococcus horse γ-globulin in rabbit antisera to the antibody (horse).

It is not always necessary, however, nor is it necessarily an advantage, to study the immunological behavior of single antigens, as Irwin and his collaborators have shown in their intricate but clearly defined studies of the numerous gene-linked antigens of avian and mammalian red cells. Another fruitful immunological approach to genetic problems has been made by Tyler in his studies of the agglutination of sperm by egg substances of *Arbacia*.

The discovery of the specific polysaccharides of pneumococcus in Avery's laboratory and the recognition that these sugar derivatives are the determinants of type-

specificity in this and other groups of pathogenic microorganisms have led to far-reaching results, most of which are beyond the scope of this lecture. However, Griffith's initially almost unbelievable discovery that one pneumococcus type could be converted into another has important implications, not only in carbohydrate chemistry and bacteriology, but in general biology and genetics as well. As many of you know, pneumococci of Type I, for example, may be degraded to a form devoid of type-specificity and then converted, theoretically, at least, back to the original type, or into any one of some forty-odd other types. This was originally accomplished by growing the degraded cells in the presence of a heat-killed suspension of pneumococci of the type into which the living cells were to be converted. Studies by Avery, Dawson and Alloway showed, however, that certain extracts of type-specific pneumococci contained a substance or substances responsible for this conversion, and that the type-specific carbohydrates themselves were not the determining factors. Thus, any pneumococcus cell is potentially able either to synthesize, one at a time, nearly fifty different specific polysaccharides or may be so influenced by a series of substances that such varied syntheses become possible. The immunochemist must leave it to the geneticist to decide whether or not these processes are true mutations, but I am happy to say that Avery is continuing the study of the transforming principle, and the eventual elucidation of its nature is certain to throw light on this and many other questions.

These are merely a few of the instances in which immunological and immunochemical methods have provided an insight into biological mechanisms. To give a more complete summary would carry me far beyond the allotted time, but I hope you will recall other examples which I would have liked to mention. More important, however, I hope that those of you who may have required this reminder of the possibilities of these powerful tools will consider them as aids in the solution of present and future biological problems.

EVOLUTION OF THE HUMAN BLOOD GROUP FACTORS[1]

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THE discovery of human blood groups by Landsteiner in 1900–01 served to explain unexpected fatal reactions resulting from transfusions of blood from one human being to another, only to pose the question, still unanswered, as to the nature of the blood group differences, their origin and function if any. This problem has become more acute during the past two decades with the discovery of additional agglutinogens in human blood, M, N, P and Rh, so that it is now possible without much difficulty to classify individuals into 72 classes, depending on what combination of factors is present in the blood.[2] Because of the limited space the discussion will be limited to the original four blood groups, the three M–N types, and the more recently discovered Rh factor.

The classic blood groups depend on the presence or absence of two agglutinogens A and B in the cells and two corresponding isoagglutinins α and β in the serum. The groups O, A, B and AB are determined by the four possible combinations of the two agglutinogens, A and B. Another characteristic property is the reciprocal relation between agglutinogens and agglutinins, those isoagglutinins invariably being present for the agglutinogens absent from the cells (Landsteiner's rule). Other striking features are the hereditary transmission of the groups by means of three allelic genes, *A*, *B* and *O*, and the differences in the distribution of the groups in different races. The most outstanding examples of the latter are the higher frequency of gene *A* as compared to gene *B* in white races in contrast to Asiatic peoples where gene *B* predominates, while in certain more primitive peoples

[1] From the Serological Laboratory of the Office of the Chief Medical Examiner of New York City.

[2] For details of the theory and technique of the blood groups, see Wiener (1943) and Schiff and Boyd (1942).

such as Australian aborigines, American Indians, Polynesians, etc., one or two of the three genes may be practically lacking.

A number of theories have been proposed to explain the present distributions of the blood groups in white races as well as the differences among various peoples. Because of the predominance of gene *O* in almost all races, it has been suggested that group O was the original group and genes *A* and *B* appeared in man later on and increased in frequency by mutation (Gates, 1936; *cf.* Snyder, 1929). Other investigators (*e.g.*, Bernstein, 1932) believe that there were originally a number of isolated races, some exclusively or predominantly group O, others predominantly group A or group B, and that the present blood group distributions resulted from crosses between these races. Candela (1942), in fact, states that the present differences in blood group distribution, in particular the striking distributions in some primitive peoples, are merely remnants of this original condition. On the other hand, Boyd (1940) has suggested that primitive man started with a certain blood group distribution and that, as he spread to the "four corners of the earth," certain isolated groups lost entirely or largely one or two of the blood group genes, giving rise to the present differences in distributions of the groups. Which, if any, of these theories is correct is uncertain, but it might be remarked that some clues have been obtained from the extensive studies on the numerous peoples in Europe and Asia. These show a progressive decrease in the frequency of gene B as one proceeds from East to West (Hirszfeld and Hirszfeld, 1919), suggesting that group B was introducted into Europe from Asia. According to Candela (1942), this occurred during the Mongolian invasions between the fifth and fifteenth centuries, the gene being derived from the brachycephalic Central Asiatic Mongols, best represented at the present time by the Buriats and Kalmucks (*cf.* Bernstein, 1932). According to computations made by Wyman and Boyd (1935) and

Haldane (1940), if the blood groups attained their present frequencies mainly by mutation, then it would be necessary to postulate an improbably high rate of mutation, and it is more reasonable to ascribe the present distribution of the blood group genes to the effects of migration, isolation and inbreeding, and racial crossing.

I propose now to discuss the light thrown on these problems by studies in lower primates. For the discussion it is necessary to mention that in man the group factors A and B are not restricted to the blood cells but are also found in tissues, organs and secretions, except that in certain individuals (known as non-secretors) the group substances are absent from the secretions.[3] Studies on saliva, the most convenient material to test, show that this character is determined by a pair of allelic genes, *S* and *s*, located in a different pair of chromosomes from the blood group genes (Schiff and Sasaki, 1932). Also, in groups A and AB subgroups have been identified, based on the existence of two main varieties of A agglutinogen, A_1 and A_2, determined by two corresponding allelic genes. Studies on the distribution of subgroups and secretor character to date have been relatively few, but the results already obtained indicate their value as additional means of tracing racial relationships.

In the first systematic study on anthropoid apes, Landsteiner and Miller (1925a) succeeded in demonstrating that the blood of chimpanzees, orang-utans and gibbons could be divided into four groups indistinguishable serologically from the human blood groups, while such blood groups apparently did not exist in monkeys or lower animals. These results are in accord with the close kinship between man and apes and at the same time furnished a striking instance of biochemical evolution. Moreover, they seemed to cast further doubt on the mutation theory already discussed, unless one was willing to assume the occurrence of parallel mutations in man and ape.

[3] In group O individuals, the two types are distinguished by testing the saliva with so-called anti-O sera.

Subsequent studies by Landsteiner (1928a), Troisier (1928) and others served to confirm and amplify these findings and at the same time revealed striking differences among the apes in the distribution of the groups. Of 92 chimpanzees examined, 81 belonged to group A, only 11 to group O, none to group B or group AB. The orangs and gibbons, on the other hand, all belonged to groups A, B or AB, so that gene *O* was apparently lacking from these species. The lack of adequate information concerning gorillas is understandable, but recently material from a dead gorilla was made available to Candela and myself. The blood serum of this animal contained anti-A but not anti-B agglutinins, but the blood cells failed to react distinctly with either anti-A or anti-B sera, in apparent conflict with Landsteiner's rule as it holds in human beings. It then occurred to us to test the salivary glands, and in this way the presence of the B substance was readily demonstrated, accounting for the absence of anti-B agglutinins from the serum (Candela, Wiener and Goss, 1940). Subsequently, additional gorillas were grouped by testing their saliva and urine; interestingly, all 13 lowland gorillas (*G. gorilla*) tested belonged or were related to group B, while 2 mountain gorillas (*G. berengei*) both belonged to group A (Candela, 1940).

While the older investigations on lower monkeys did not reveal the presence of groups corresponding to the human blood groups,[4] tests on their blood sera demonstrated the existence of certain regularities. For example, the sera of rhesus monkeys always contain anti-A but not anti-B agglutinins, while sera from vervet monkeys contain anti-B but not anti-A (Landsteiner, 1928b). Tests on the blood cells failed to account for this phenomenon, but in view of the results obtained in gorillas, it occurred to the writer that the explanation for the phenomenon might be found by testing the organs and secretions. Wiener, Candela and Goss (1942) then found that

[4] It should be mentioned, however, that Landsteiner and Miller (1925b) did demonstrate the presence of B-like antigens in the erythrocytes of New World monkeys but not in Old World monkeys.

the secretions and organs of rhesus monkeys regularly contained the group substance B. By extending the tests to other monkey species, it was established that in general, in monkeys and gorillas as in other apes and man, Landsteiner's blood group rule holds, but the reciprocal relationship exists between agglutinins in the blood serum and antigens in the organs and secretions, rather than the blood cells. Accordingly, group substances are present in organs and secretions in lower primates, while their presence in the erythrocytes appears to be a more recent development in evolution.

Gene *O* appears to be rare in monkeys as well as apes, because in the admittedly small series of tests carried out to date only a single monkey giving reactions corresponding to group O was encountered, and in this instance one can not entirely exclude the possibility that the monkey was a non-secretor. This is in striking contrast to the situation in man where gene *O* has the highest frequency, and may suggest that instead of O being the original group, genes *A* and *B* came first and that gene *O* arose later by mutation. If this assumption is correct, then it is more likely that gene *O* arose from gene *A* than from gene *B*. This follows from the existence in some races of man and in chimpanzees of groups A and O alone, to the exclusion of gene *B*. Moreover, the varieties of A agglutinogen, A_1, A_2, A_3, . . . form a graded series which in serological tests react progressively less intensely with anti-A sera and more intensely with anti-O sera.[5]

The distribution of the blood groups in monkeys is similar to that of apes in that in monkeys of a single species not all four groups are represented. In man, also, in Paleolithic times when man was presumably a comparatively rare animal, isolation and inbreeding of small numbers of individuals may have occurred, giving rise by chance to populations lacking one or more of the blood groups. The present distributions of the blood group genes, in the writer's opinion, can be explained in part

[5] This graded series of genes has also been discussed by Hirszfeld (1938), who, however, believes that gene *A* arose from gene *O*.

by crossing of two or more such populations with different blood group distributions (as Bernstein and Candela believe), and in part to subsequent migration to distant parts of the world of small groups (as Boyd believes) with chance loss of one or more of the blood group genes. As Wyman and Boyd remarked, this would explain the similarity in blood group distribution of peoples geographically distant from one another.

It may be well to point out at this time that the fact that blood or secretions from apes, monkeys and man give identical or similar reactions with anti-A and anti-B testing sera does not necessarily establish the presence of identical substances in these species, but merely of chemically related substances. Though it is difficult to test the point, presumably the substances in apes which give group-specific reactions are more closely related to the human group substances than those present in monkeys. In fact, substances serologically related to the group substances A and B have even been found in the blood, organs and secretions of certain lower mammals. To be sure, in these lower species the reciprocal relationship between group substances and agglutinins in the sera appears to be less striking, but it is not entirely certain how significant this is, because the tests to date have mostly been carried out with reagents prepared from human blood. Incidentally, the blood group rule has practical application, for example, when selecting rabbits for the production of immune anti-A sera, in that rabbits lacking substances giving A reactions produce far more potent antisera than those possessing such substances.

Studies on the properties M and N (Landsteiner and Levine, 1928a, b) of human erythrocytes have furnished further data concerning biochemical evolution. In man these properties are transmitted by a pair of allelic genes, *M* and *N*, located in a different pair of chromosomes from *A*, *B*, *O*, and they give rise to three types of blood, M, N and MN. Unlike A and B, however, factors M and N appear to be confined to the erythrocytes. In most white

races the distribution of the types is approximately 30 per cent. M, 20 per cent. N and 50 per cent. MN, so that gene *M* is slightly more frequent than gene *N*. American Indians and Eskimos are characterized by a high frequency of gene *M*, while Australian aborigines and Ainu have a high frequency of gene *N*. Besides confirming the relationship between American Indians and Eskimos, on the one hand, and Ainu and Australian aborigines, on the other, these results suggest that the present distribution of the M-N types among white individuals may have arisen from crossing some time in the past of two or more races, some with high frequencies of gene *M*, others with much gene *N*. These results, accordingly, are in line with our conclusions from studies on the factors A and B.

Factors corresponding to M and N have not been found in the blood of lower animals. Their presence in the blood of anthropoid apes was first reported by Landsteiner and Levine (1928b), but for some time thereafter contradictory results were obtained with monkey blood. In testing for property M and N, it occurred to the writer to examine monkey and ape blood with a variety of antisera. It was then found that the irregular results previously reported were due to qualitative differences in the M antisera, even though they all gave parallel reactions on human blood; for example, some anti-M sera agglutinated the blood of rhesus monkeys while other anti-M sera gave no reactions. Tests were then carried out by Landsteiner and Wiener (1937) and Wiener (1938) with a variety of anti-M sera on a number of species of apes and monkeys, with the results given in Table 1. It was found that several different M-like antigens exist in the blood of monkeys and apes, so that the species could be arranged in a graded series based on the resemblance of their blood to human M blood. As expected, the blood of anthropoid apes resembled human M more closely than monkey blood, chimpanzees' blood being the most like the human. Among the monkey species, M agglutinogens were regularly found in the Cercopithecidae but were found in only

one of the species of Platyrrhinae (and in this case the blood reacted only with a special serum), suggesting that the former are higher in the evolutionary scale.

In tests made with anti-N sera, positive reactions have thus far been obtained only with blood from chimpanzees (Landsteiner and Levine, 1928b; Wiener, 1938). Not all anti-N sera react with chimpanzee blood, indicating that the N factor in the ape blood (like the M factor) is related

TABLE 1

M AGGLUTINOGENS IN MONKEY BLOOD

Source of Blood Specimens	Anti-M Testing Fluid*					
	M5	M1	M21	M35	M2	M82
Human M	+++	+++	++±	++±	++±	++±
Human N	0	0	0	0	0	0
Chimpanzee	+++	+++	+++	++	+++	±
Old World Monkeys (Cercopithecidae)						
Sphinx Baboon	+++	++	++±	0		0
Drill Baboon	+++	+++	++±	(±)	(+)	(+±)
Chacma Baboon	+++	+++	++±	0	tr.	0
M. rhesus	+++	+++	++±	(+±)	±	0
Java Macaque	+++	+++	+±	0	0	0
Sooty Mangabey	+++	+++	++±	tr.	±	±
Green Monkey	+++	+++	0	0	0	0
New World monkeys (Platyrrhina)						
White Spider Monkey	++±	0	0	0	0	0
Black Spider Monkey	±	0	0	tr.		0
Wooly Monkey	0	0	(±)	0	0	0
Brown Ringtail (Capuchin Monkey)	0	0	0		0	0
Moss Monkey	0	0	f.tr.	±		0
Lemur	0	0	0	0	0	0
Average Titer of Testing Fluids	64	64	32	24	16	16

Of several species two individuals were tested, of brown ringtails 4, of *Macacus rhesus* 45.

Reactions placed in parentheses were found not to be removed by absorbing the sera with human M blood. In addition there are a number of weak reactions which were not tested with human M blood but probably belong to the same category.

* These were prepared from the immune sera in the usual way, by diluting with saline and then absorbing with packed human N cells.

to but not identical with the corresponding property in human blood. Interestingly, all chimpanzees thus far tested have been found to possess both M and N, while if these were contrasting characters as in man, only half of the apes should have the two factors together. Perhaps, in these apes the reactions for M and N may both be due to a single chemical substance and the discrete properties M and N of man may possibly have arisen by

mutations in opposite directions from some such common antigen.

Based on the demonstration of M-like agglutinogens in monkey blood, Landsteiner and Wiener (1937) attempted the production of anti-M sera by immunizing rabbits with rhesus blood, and found that potent anti-M immune sera could be obtained in this manner. Similar results were obtained by Wheeler and Stuart (1939). It was considered that in the same way it might be possible to obtain type specific immune sera for human blood against factors hitherto unknown, if such factors were present in monkey blood. In fact, Landsteiner and Wiener (1940, 1941) then succeeded in obtaining anti-rhesus immune sera (first in rabbits, then much more readily in guinea pigs), which reacted with about 85 per cent. of bloods from white individuals. The factor present in human blood of the former type was designated as Rh, to indicate the manner in which it was first detected. The agglutinogen Rh was subsequently found to be of clinical importance, on account of its role in the pathogenesis of intragroup hemolytic reactions (Wiener and Peters, 1940; Wiener, 1941) and in a disease of the newborn known as erythroblastosis fetalis (Levine *et al.*, 1941).

As already mentioned, it has not yet been possible to ascribe any physiological function to the blood group factors. The Rh factor is the first thus far discovered which has proved to have a definite, though weak, selective effect, because it can be responsible for stillbirths or neonatal deaths, as pointed out above. In the matings in question, the mother is Rh-negative, the father Rh-positive, and the fetus in utero Rh-positive, having inherited the factor from the father. Due presumably to some defect in the placenta, some of the fetal blood passes into the maternal circulation, and in susceptible women this results in the production of anti-Rh isoantibodies. The Rh isoantibodies then filter through the placenta and destroy the blood of the fetus either in utero or shortly after birth, giving rise to symptoms of the disease. It may be of

interest to mention that in one of ten to twelve matings the mother is Rh-negative and the father and child Rh-positive, while the incidence of the disease is only about 1 in 400, indicating that only about one in forty women is capable of being sensitized.

It can be shown (Wiener, 1942) that in populations where the genes *Rh* and *rh* are equal in frequency—Landsteiner and Wiener (1941) have shown that the Rh factor is transmitted as a simple mendelian dominant by a pair of allelic genes, *Rh* and *rh*—the death of infants and fetuses with erythroblastosis will have no effect on the relative distribution of the genes. When the incidence of the genes is unequal, however, there will be a definite selective action against the less frequent one. It is of interest to note that certain populations have been found, *e.g.*, American Indians (Landsteiner, Wiener and Matson, 1942) and Chinese (Levine, 1942), in which the Rh-negative type is practically lacking. It is difficult, however, to explain the high incidence of the recessive gene (almost 40 per cent.) in white populations. Gates's theory proposed for the blood groups if applied to the Rh factor, that the present distribution was built up by repeated mutations from *Rh* to *rh*, can immediately be excluded because the selective action of isoimmunization would certainly nullify the effect of such mutations. Again we must fall back on the theory of two or more original races, predominately Rh positive or negative, respectively, the present distribution being explained by crossing of these races some time in the past.

Some remarks on the relationship between species-specific and type-specific antigens are pertinent. It is significant that certain factors characteristic of the individual in man are shared by all members of other species. For example, the so-called F_A antigen is present only in human blood of groups A and AB but appears to be shared by all sheep, and M antigens have been found in all rhesus monkey blood thus far examined. In doves and pigeons, Irwin *et al.* (1936, 1937, 1940) have demonstrated that the

species-specificity of the blood is determined by a number of agglutinogens shared perhaps by all members of the same species, and in species crosses the genes for these agglutinogens undergo independent assortment giving rise to a large number of individual blood differences in subsequent hybrid generations. In this connection, as already mentioned, members of the species of monkeys thus far examined have been found to belong predominantly to a single blood group, suggesting again that the four blood groups possibly arose by crossing (*cf.* Landsteiner and Miller, 1929a).

In conclusion, the observations on the blood groups factors in apes and monkeys indicate that biochemical evolution has roughly paralleled morphological evolution. While no function has yet been established for the individual blood antigens, the observations on isoimmunization in pregnancy are of interest. No evidence is available as to whether in certain cases such a mechanism might perhaps interfere with the viability of the offspring of species crosses. Future investigations will undoubtedly throw further light on this intriguing problem.

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IMMUNOGENETIC STUDIES OF SPECIES RELATIONSHIPS[1]

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NEARLY all biologists are aware that the branch of science called "immunology" had its origin in the study of reactions to infectious diseases. The concept that each disease was the result of the interaction of the host and a particular infecting agent was followed shortly thereafter by the knowledge that there was a specificity in the resistance produced by an infection. Studies to determine the nature of this striking phenomenon gave birth to the science of immunology. Whether this once lusty infant is now approaching vigorous maturity is not a subject of discussion at this time. However, we may definitely state that this one-time infant prodigy has long since weaned itself from its mother-science, bacteriology, and has embarked on an independent career which has given much, and promises more, in explanation of the specificities of biological reactions.

Another egg cell, which later developed into the scientific discipline now called "genetics," was fertilized at about the same time as that of bacteriology, the mother-science of immunology. The infant developing from this fertilization was given no nourishment at birth and promptly went into a state of coma which lasted for several decades. Three "wise men" of our own time discovered the infant in this state and, upon receiving proper attention and adequate nourishment, it made a phenomenal growth. There have been a few persons who predicted a "bad end" for the youngster, but *he* has managed to survive and *their* voices are stilled.

Actually, the growth periods of these two branches of science are relatively equal. Moreover, while immunol-

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ogy sprang directly from bacteriology, genetics had two parents, botany and zoology. A genealogist would of course remind us that these parent branches are but parts of a single stem, biology, making the relationship of two of the disciplines which are being discussed to-day as that of first cousins. Certainly both of these cousins have contributed greatly to our conception of specificity in biological reactions, a goal towards which biological research in general is ever striving. If each of these by itself is able to make significant contributions to our understanding of biological phenomena, surely much could be anticipated from their combined efforts.

One of the first joint attacks of these two branches of science was made in the study of antigenic characters in the red blood cells of humans, the discovery of which was made by Landsteiner (1900, 1901). The same worker was also first to use the antigens of the blood cells in a comparison of the relationships of two species and their hybrids (Landsteiner and Van der Scheer, 1924), in contrast to earlier immunological studies of species relationships of animals in which serum had been employed almost exclusively. These findings, in conjunction with those obtained by Heidelberger and Avery (1923, 1924) which showed that the immunological specificities of the different types of pneumococci were amenable to a chemical explanation, suggested some years ago that the combined use of these two disciplines might well yield fruitful results with suitable experimental material. Although certain plant material appeared at the outset—and still does—to be favorable for the proposed experiments, the various species and backcross-hybrids which Professor L. J. Cole had previously produced at the University of Wisconsin seemed tailor-made for such a study. All these were generously placed at our disposal.

In making a study of genetic relationships between species it would undoubtedly be of advantage to use characters whose expression is not affected by different genetic complexes. The antigens of the red blood cells

appear to be an example of such characters. The relatively small number of cell divisions obtaining between the fertilized egg and the laying down of the mother cell of the tissue which forms the blood cells is one line of evidence which makes it reasonable to assume that the cellular antigens are the more or less direct products of their respective causative genes. Indeed, Haldane (1938) has postulated that "the gene is a catalyst making a particular antigen, or the antigen is simply the gene or part of it let loose from its connexion with the chromosome." However, the findings that antigenic characters, either in species hybrids (Irwin, 1932) or within a species (Thomsen, 1936), may be the result of complementary action of genes, show that interaction is sometimes possible between genes affecting these substances. Hence a sweeping statement can not be made that there is but one step from gene to antigen. Nevertheless, other than these few examples of complementary interaction, it appears that in general the genes with antigenic effects produce the same result irrespective of the other genes present. As Wright (1942) has stated, "Here we seem to have epigenesis at its simplest."

The technical procedures used in our laboratory in making comparisons of various species of birds have been described elsewhere in detail (Irwin and Cole, 1936; Irwin, 1939). Briefly, it can be said that a definite differentiation of the cells of any pair of related species of pigeons and doves has been possible only after the antiserum to one has been absorbed by the cells of the other. For example, when antiserum to Pearlneck (*Streptopelia chinensis*) has been exhausted of part of its content of antibodies by mixing it with an excess of the cells of Ring dove (*St. risoria*), it becomes a "reagent" which will agglutinate the cells of Pearlneck, but not those of Ring dove. The antigens in Pearlneck reactive with this reagent have been called the "species specific" characters of Pearlneck. This procedure has been portrayed diagrammatically (Cumley and Irwin, 1941), and a schematic

representation of the antigens of Pearlneck, Ring dove and their F_1 hybrid as determined by these methods has been made (Irwin and Cole, 1936).

By virtue of the results from comparisons of the cells of many pairs of species of birds by such tests one may conclude that, for any pair of related species, each possesses two kinds of cellular antigens, *viz.*, (a) those which are peculiar to one or the other of the species, and (b) those which are shared by both.

In so far as hybrids have been produced between various species, they invariably have been found to possess all or nearly all the cellular components particular to both parental species, and all those shared by the parents (except for differences between the hybrids attributable to heterozygosity of one or both parents for species-specific or common components, or both). Also, hybrids between certain species only, not all species-hybrids, possess an antigenic complex which was not found in either parental species. This new or "hybrid" substance presumably is produced by the interaction of genes which in each parent produce only species-specific antigens, if any at all.

On the assumption that the *species-specific* and *common* antigens are gene-determined, definite ratios of the antigens specific to one species would be expected in the offspring of backcrosses of the species hybrid to the other parental species. (Obviously, the genes which produce the antigens peculiar to either species are simplex in the species-hybrids.) For example, if there were but one cellular component which differentiates the first from the other species, two kinds of progeny from mating the species hybrid to the second species would be expected in equal numbers—those with and those without the character. If there were two such characters, and their causative genes were on separate chromosomes, approximate equality for four types of backcross progeny from the same kind of mating would be expected—those with both components, those with only one, those with the other

and those with neither. With any number of antigens, the number of types of offspring in the first backcross generation would be 2^n, in which n equals the number of species-specific antigens.

Antigens of Red Blood Cells

A. *Segregation of species-specific antigens in backcross progeny.* To use a specific example, offspring have been obtained by backcrossing, to Ring dove, species-hybrids from matings between Pearlneck and Ring dove. As yet only Pearlneck males have produced hybrids in this cross, and only hybrid males have produced backcross progeny. These circumstances rule out the possibility of cytoplasmic influence on the segregating Pearlneck antigens, since, as is generally admitted, any such influence would be transmitted through the cytoplasm of the egg.

Approximately ten cellular antigens peculiar to Pearlneck have been isolated in unit-form as a result of their segregation in progenies of various backcross generations (Irwin, 1939). These cellular characters have been called d–1, d–2, d–3 . . . d–12, the letter "d" indicating that the characters are found in doves. The genetic test of the unitary nature of each of these antigenic components has been that any backcross bird containing only one of these specific Pearlneck characters should produce but two kinds of progeny in matings to Ring dove; *viz.*, those with and those without the particular substance. That each of these characters, as carried by the backcross birds, is definitely different from the others may be shown by an extension of the immunological technic which permits the differentiation of the cells of one species from those of another. Such tests have been described previously (Irwin, 1939) and also presented diagrammatically (Cumley and Irwin, 1942a).

Although the proportions of backcross offspring with and without the respective characters peculiar to Pearlneck approximate those expected if each were the product

of a single gene, it is possible—and to us it seems highly probable—that most if not all of these specific antigens may be the result of the combined action of several linked genes on the particular chromosomes. Usually in genetic parlance a heritable character which seemingly behaves as a unit is said to be produced by a single gene until evidence is obtained that more than one gene is involved. However, despite the resemblances the unit characters of each of these specific characters, in terms of genetic ratios, it is known that some of them are complex antigenically, and probably also genetically. One explanation of the known complexity of at least two of these antigenic components (d–6 and d–11) of Pearlneck is that two or more genes are acting together to produce each (Irwin and Cole, 1940). Unpublished data indicate that a parallel situation obtains for others of the characters.

What really has been accomplished by the genetic segregation of these antigens in the backcross generations parallels the fractionation of the antibodies in an immune serum by antibody-absorption. Furthermore, the experimental results are in complete accord with the concept long held by immunologists that there are multiple antigens in cells, and that the various antibodies engendered by an animal during immunization with these cells are highly specific for the respective antigens.

Some unpublished evidence indicates rather strongly that the sum of these ten or so cellular characters now demonstrable equals the total complex of specific Pearlneck substances which are found in the species-hybrids. One can not, however, eliminate the possibility that antigens particular to Pearlneck other than these ten may exist, but, if so, it is probable that they are not detectable at the threshold of reaction (*i.e.*, at the dilution of the reagents) at which the tests have been made. The demonstrable characters (specific to Pearlneck) may then be spoken of provisionally as "major" characters, in contrast to others detectable only at lower dilutions of the antiserum, which might be called "minor" characters.

These two species are readily differentiated by certain visible external characteristics. To date, critical tests have not been made of a possible correlation in backcross generations between the antigenic and the external characters that distinguish Pearlneck from Ring dove. Such a study does not appear promising at the moment, primarily because most of the visible characters of these two species intergrade to a considerable extent. An interesting observation has been made, however, by Shrigley (1940), that various types of abnormalities of the sperm, occurring infrequently in both these species, were markedly increased in the species hybrids. Furthermore, there was a definite tendency for backcross individuals which possessed a complex of antigens peculiar to Pearlneck to have a higher proportion of abnormal sperm than did the backcross birds lacking these properties. The backcross birds in the latter class were biochemically more like Ring dove than were the others, as were the proportions of abnormalities of their sperm. These results suggest a disharmony between at least certain of the genes of Pearlneck and those of Ring dove, which is reflected in the sperm. Other studies dealing with morphological and physiological differences between these species are indicated.

It is unfortunate that cytological studies of these backcross individuals, which might show a correlation of chromosome behavior with the cellular antigens, appear at the moment to be impractical, if not impossible. However, on the assumption that each of the ten characters in Pearlneck is produced by one or more genes on as many chromosomes, an estimate may be made of the relative proportion of the chromosomes of Pearlneck bearing genes for "major" cellular substances either specific to itself or common with Ring dove. The best cytological evidence suggests that there are approximately 30 pairs of chromosomes in pigeons and doves (Painter and Cole, unpublished data). Therefore, if one or more genes on at least many of these have a detectable effect on the

antigens of the blood cells, about one third of the total will carry genes with "major" effects distinguishing Pearlneck from Ring dove; all others with such effects will produce components *common* to the two species. Naturally, the proportion of the total chromatin material effecting either species-specific or common components need not necessarily parallel the proportion of the numbers of chromosomes with such diverse effects.

On the assumption that each of the genes producing cellular antigens particular to Pearlneck, as well as each of those producing the *common* substances, might have an allele with a different effect, the number of different combinations of antigenic characters within the species appears to be almost without limit. The greatest number of antigens known at present for any species has been found in this laboratory in cattle (Ferguson, 1941; Ferguson *et al.,* 1942), in which species more than 30 cellular substances have been demonstrated as probable units. The majority of these antigenic characters within the one species of cattle are detectable only at lower levels of reactivity (*i.e.,* at lower concentrations of antisera) than those which differentiate Pearlneck from Ring dove. They would then be classified as "minor" rather than as "major" characters, according to the grouping proposed earlier. (Unless the different cellular antigens which distinguish one species from another are of a different "order" than at least most of those which distinguish individuals within a species, the very anomalous situation presents itself in which individuals of the same species might differ in more antigens than do different species. That is, it has been found that the cells of Pearlneck differ from those of Ring dove by probably ten cellular antigens, whereas the cells of individual cattle—the same species—theoretically may differ by as many as 30 such antigens.) The genetic relationships of each of these 30-odd characters of cattle cells to the others have not yet been worked out in detail, but it seems probable that a marker has been provided for at least one member of

most of the 30 pairs of chromosomes of cattle as determined by Krallinger (1931). Furthermore, since no two of these antigens seem to have a simple allelomorphic relationship, the number of their possible combinations is well over a billion (2^n in which n equals 30, the number of different cellular characters). Thus, for this species and undoubtedly for many others, the biochemical specificity of the individual shows definite promise of being a reality, and not simply a possibility as yet unproven.

Furthermore, studies by various workers (Landsteiner and Levine, 1932; Todd, 1930, 1931, 1935; and unpublished data from this laboratory) on the cells of the chicken indicate that a very large number of cellular antigens is present in that species also, making more plausible the postulate that one or more genes on at least the majority of the chromosomes of a species may have effects on the cellular antigens.

Indeed, if a gene is a chemical entity, and if each gene is present in every cell of the body—at least in all those which have a nucleus—it might well be argued that every gene should be represented by an antigen in the red blood cells of birds, since they possess a nucleus. On such a basis the upper limit of the number of antigens in a given kind of cells would be the number of genes which they contained. However, it is conceivable that many more genes may have antigenic effects on the cells than can be readily detected. That is, on the assumption that the immunological reactions are surface phenomena, there may be many antigens which are not expressed at the surface of the cells, and hence would not react with an immune serum. What appear to be examples of this kind are assumed to exist in bacteria (Topley, 1933). Also, in human cells, a property (T) is detectable only after the action of environmental influence on the corpuscles (Friedenreich, 1938).

Several pertinent questions might be raised at this point. For example, to what extent may antigenic differences between individuals in either Pearlnecks or Ring

doves have influenced these findings? That is, do any Ring doves possess cellular characters which are supposedly peculiar to Pearlneck? At present, this question may be answered in the negative. More than five hundred Ring doves from various sources have been tested at one time or another over the past ten years, without a suggestion that any one of these possessed even one of the specific Pearlneck characters.

B. *Relationships of antigens of one species to those of two others.* It is of interest also to inquire whether any of the cellular antigens which distinguish Pearlneck from Ring dove are found in any other species. Should they be, the various single characters of Pearlneck would serve as "testers" to determine what combination of these was shared between Pearlneck and a third species, to the mutual exclusion of Ring dove. In such comparisons, a third species, the Senegal (*St. senegalensis*), has been of special interest because not only do its cells appear to contain nearly all the substances *common* to Pearlneck and Ring dove but, in addition, its corpuscles possess at least a part of each of the cellular characters peculiar to Pearlneck, not in Ring dove (Irwin and Cole, 1940). From such results it may be stated that at least to this extent the cells of Senegal may be differentiated from those of Ring dove.

If Senegal cells contain *all* the specific parts of Pearlneck cells, and nearly all those common to Pearlneck and Ring dove as well, it would be practically impossible to distinguish Pearlneck cells from those of Senegal. However, reciprocal comparisons of the corpuscles of these two species (Pearlneck and Senegal) have shown that a distinction between them may be as readily accomplished as between either and Ring dove. Clearly, then, not all the specific components of Pearlneck are contained *in toto* in Senegal. The actual tests (Irwin and Cole, 1940) have revealed that Senegal shares with Pearlneck all of 7 cellular antigens (d–1, d–2, d–3, d–4, d–5, d–7 and d–9), but only a part of three others (d–6, d–10 and d–11). More

recently another Pearlneck character (d–12) has been recognized, which is not shared with Senegal *in toto*, if at all.

The relationships so far described between the specific cellular characters of Pearlneck and the Senegal complex are based entirely on findings by immunological technics. On genetic grounds, if Pearlneck differs from Senegal mainly if not entirely in three cellular antigens (d–6, d–10 and d–11), these should segregate in Mendelian fashion in backcross offspring, following the cross of Pearlneck and Senegal. At the time such tests were made, it was recognized that Pearlneck shared with Senegal a part of d–6 and d–11, and in the first backcross generation (to Senegal) there appeared the four kinds of individuals expected; viz., those with both d–6 and d–11, those with either d–6 or d–11 and those with neither (Irwin and Cole, 1940). (There was also a third character segregating, which probably was d–10, although the differentiation of d–6 and d–10 is not always possible.) Thus the genetic segregation in the backcross offspring entirely confirms the relationships proposed by the use of direct immunological tests.

This resemblance but not identity of heritable antigens in Senegal to the three single characters of Pearlneck (d–6, d–10 and d–11) may be explained in either of two ways, or by a combination of these. (1) If each of these three antigenic characters of Pearlneck is produced by two or more genes on each of the chromosomes, there may be genes in Senegal identical with some, but not with all, of the respective genes of Pearlneck producing the three characters. On this explanation only that part of each of these three antigens of Pearlneck would be shared with Senegal, for which Senegal possessed homologous genes. For example, if the d–11 character of Pearlneck were the result of the joint action of but two linked genes, Senegal might have a gene homologous to one of these. That species would thereby have a part, but only a part, of the Pearlneck character d–11. (2) On the other hand,

if each of these three characters peculiar to Pearlneck were the result of the action of a single gene, the only reasonable conclusion would be that in Senegal there were genes producing similar but not identical chemical effects. The genes themselves in each of the two species therefore would presumably be related, but not identical, in their own chemical constitutions. Several examples of this kind of relationship appear to obtain between the known antigens of human blood cells, supposedly produced by single genes, and those of the anthropoid apes and lower monkeys (Landsteiner and Miller, 1925a, 1925b; Landsteiner and Wiener, 1937; Wiener, 1938, 1943).

It may be concluded from the above evidence that the biochemical relationships between Pearlneck and Senegal are much closer than those between Pearlneck and Ring dove. It was stated earlier that approximately 10 of the probable 30 pairs of chromosomes of Pearlneck carry one or more genes that produce cellular antigens which differentiate Pearlneck and Ring dove. On this same basis, only three, or possibly four, chromosomes of Pearlneck carry genes which distinguish its blood cells from those of Senegal.

These findings suggest still another means of assaying relationships between Pearlneck and Senegal in contrast to Ring dove. The experimental results have shown that Pearlneck shares *in toto* with Senegal seven of the ten or so cellular components which distinguish Pearlneck from Ring dove. Should the cellular characters which differentiate Senegal from Ring dove be obtained in unit form, will there be seven of these characters indistinguishable from and therefore supposedly identical with the seven known characters of Pearlneck? Or will they exist in different combinations in Senegal, as would be expected if in that species there were different linkage relationships of the causative genes? Only a partial answer to these questions can be given at present. From unpublished data, it does appear that two specific antigens (d–3, d–4) of Pearlneck are also units in Senegal. However, at

least one antigen of Pearlneck (d–1) appears in Senegal to be inherited as two substances rather than as one, indicating that in Pearlneck this character is caused by the joint action of at least two linked genes. Studies of the reciprocal relationships of the unit antigens of Pearlneck and Senegal, respectively, not in Ring dove, are being continued and will be reported in detail elsewhere.

These results and those from other comparable experiments (Irwin *et al.*, 1936; Irwin, 1938) allow the general statement that the genetic relationships of the blood cells of various species may be determined by appropriate immunological tests. Naturally, these will not reveal the number of antigenic characters differentiating one species from another. In this connection, comparisons of eleven species of the genus Columba (Irwin and Cumley, 1943) have shown that the antigens peculiar to one species in comparison with another were usually shared, at least in part, with several other species. Also, for certain species in relation to others it was found that a given species might share (a) a complex of cellular characters with one species, (b) the same complex or the same complex plus additional antigens with another species, (c) all the components of the second species plus others with a third, etc. Each of the species studied, in its relationships to the others, appears definitely to be an entity. The cellular characters of each species interlock in intricate but somewhat dissimilar patterns with those of the others. Despite differences in interlocking relationships towards other species which might account in part for the differentiation between two species, it appears possible for a species—but not necessarily all species—to contain antigens (therefore genes) which were not found in any others of those tested.

Antigens in the Serum

The question naturally arises now as to whether the serums of these species have comparable specific antigens and, if so, whether the same genes have effects in both

serum and cells. As is well known, a considerable body of experimental data has accumulated on the interrelationships of the serum antigens of many species, following the classical researches of Nuttall (1904). Although it undoubtedly has been tacitly assumed by many of the investigators that these experimental studies revealed some sort of genetic relationships between the groups under comparison, only recently has definite evidence been obtained that the species-specific antigens of the serum are gene-determined.

As was found in attempts in our laboratory to differentiate the cells of closely related species of birds, so also was it found to be impossible to differentiate the serums of these same species by the use of untreated antiserums. (The antiserum is diluted in determining its agglutination titer, whereas in the usual tests to determine the precipitation titer of an antiserum, the antigen is diluted, and the antiserum is used undiluted or at a constant dilution. To many workers it seems illogical to apply the same reasoning to the results obtained from such tests as can be used if the antiserum, not the antigen, is diluted.) But if an antiserum prepared in a rabbit against the serum antigens of one species was absorbed by the serum of another species, a "reagent" was prepared which would react with the homologous, but not with the absorbing serum. Thus, in our experiments the differentiation of the two kinds of serums has been on the basis of the presence or absence of a reaction. Following the same terminology used for the relationships of the cellular characters of two species, the reactive components of the homologous serum with the absorbed fluids have been termed "species-specific," and those for which antibodies have been removed in the absorption have been called the "common" constituents of the serum of one species in relation to the other. This terminology has no implications as to the structure of the serum antigens, which presumably are proteins.

A segregation of the serum antigens specific to Pearlneck, comparable to that noted in cellular antigens, has been observed in the progeny of successive backcrosses to Ring dove. Although these specific serum antigens have not yet been obtained in unit form, as have the cellular antigens, it is reasonably certain that there are three or more distinct serum antigens peculiar to Pearlneck (Cumley and Irwin, 1942, and unpublished data), whose distribution in the backcross families simulates that expected if each were the result of the action of one or more genes on a particular chromosome. It is significant that there was independent segregation of the antigens of the cells and serum, so that a single backcross bird might have Pearlneck specific antigens in either cells, serum or in both, while both kinds of antigens might be entirely lacking in another. Therefore the possibility is eliminated that the antigens of the serum are in the nature of disintegration products of the cells, as has been noted in the serums of some humans whose cells carry **A** or **B**, or both. (An extensive analysis of the proportions of individuals carrying cellular-like substances in their serums has been reported by Aubert *et al.*, 1942.)

Corroborative evidence of segregation of the species-specific components of the serum, and their probable genetic independence from the specific cellular components, has been obtained in backcross offspring of three other species-hybrids (Cumley, Irwin and Cole, 1941; Cumley *et al.*, 1943; Irwin and Cumley, 1942). The present evidence indicates that, for each of the various species whose species-specific antigens have segregated in backcross progeny, between three to five serum antigens distinguish the serum of the one species from that of the other. The implication follows that as many chromosomes were involved as there were antigens peculiar to a species. These findings make reasonable the conclusion that all the biochemical constituents of the serum, not only those which are species-specific, of these and other species are gene-determined.

The two kinds of antigens of the blood of Pearlneck may now be used together to give a more accurate analysis of the biochemical relationship of this species to Ring dove than is possible with either kind of antigen alone. In terms of chromosomes, then, it appears that the Pearlneck has ten chromosomes each with one or more genes with effects peculiar to itself on the cellular antigens and, seemingly, a minimum of three other chromosomes carrying genes which produce specific antigens of the serum. Thus, out of a total of approximately 30 pairs of chromosomes, Pearlneck has 13 or more with genes affecting specific antigens in either serum or cells. That is, slightly less than half the chromosome complex of Pearl-

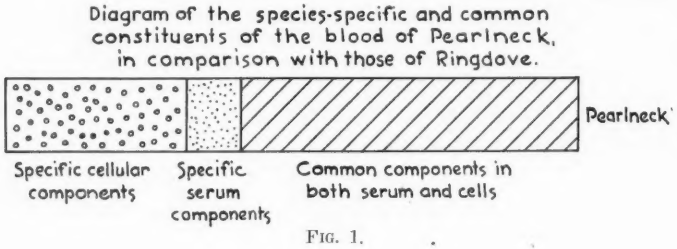

FIG. 1.

neck—but not necessarily the same proportion of the chromatin material—carries genes which differentiate its blood from that of Ring dove. A diagrammatic sketch of the summation of these different kinds of antigens of Pearlneck in comparison with Ring dove is given in Fig. 1.

Furthermore, as stated earlier, Pearlneck is more closely related to Senegal than to Ring dove, on the basis of cellular characters, differing from that species in antigens produced by genes on only three or four of the probable ten chromosomes which set it apart from Ring dove. Indeed, as explained above, a part of the effects of the genes on these three or so chromosomes of Pearlneck, carrying genes producing antigens not in Ring dove, actually are shared with Senegal, implying linkage of genes producing *common* and *specific* effects, respectively. On the other hand, in antigens of the serum Pearlneck appears

to differ from Senegal in about the same number (three), but possibly not in the same serum antigens, as distinguish it from Ring dove (unpublished data). Only six or eight of the 30 pairs of chromosomes of Pearlneck carry genes whose effects on either cells or serum distinguish its blood from that of Senegal.

Thus it may be concluded from these findings that the genes which produce the "major" biochemical differences between these species are located on a relatively small proportion (less than half) of the chromosomes of the respective species, rather than being scattered at random over most of the chromosomes. All data of this laboratory, published and unpublished, support this conclusion. If each of the antigens specific to one species is the cumulative effect of many "small" genes on a particular chromosome, the overall picture of gene-differences between species might, with some modification, fit in with current genetic theory of such differences, as discussed by Muller (1940). But if each such antigen were produced by the action of a single gene, or by a few linked genes, it hardly seems that these data would fit the theory that the differences between species depend upon multiple genes, having individually small effects.

At present no definite statement can be made as to the probable number of genes effecting any one of the species-specific antigens thus far identified. However, if any one of the cellular antigens which have been isolated as presumed units (of Pearlneck as contrasted with either Ring dove or Senegal, or of *Columba guinea* as compared to *C. livia*) were produced by the cumulative action of many genes, one might well anticipate an occasional further fractionation of the antigen as a result of crossing over. Naturally, the linkage relationships of these hypothetical small genes in the one species as compared with those in the other would determine whether there was any interference with normal synapsis and subsequent crossing over. No information is at present available on this subject. As stated earlier, the criterion for each unit

antigen has been approximate equality of birds with and without the component in the backcross offspring of an individual carrying the character. Routine tests have nearly always been made of the cells of backcross progeny carrying the antigen to see if they possessed all or only a part of the cellular component found in the parent. At present, only three possible instances of fractionation of any of these antigens, presumably following crossing over, have been noted; two of the d–1 and one of the d–4 character. No offspring were obtained from these three individuals so genetic verification of the probable fractionation of the respective antigens is not available. From the evidence at hand, it appears that crossing over involving the chromosomes carrying genes for the species-specific characters has been very infrequent in the backcrosses, if it has occurred at all. But this evidence does not permit any statement of the kind of effects—whether small or large—of the causative genes.

In each of the four kinds of species crosses in which a segregation of antigens of the serum and cells has been demonstrated, the two kinds of antigens have separated independently. Although the possibility of a loose linkage of the causative genes can not be entirely eliminated, it seems probable that the species-specific antigens of the cells and serum are produced by genes on independent chromosomes. One might wonder whether there is a mutual exclusion of linkage of genes affecting the species-specific antigens of the serum and cells, respectively, or whether the independence observed in the four different species crosses was only fortuitous. However, at present there is no known reason why there may not be linkage of genes with effects on both serum and cells. There may well be such linkage between the genes affecting the *common* components of both serum and cells in the species already tested.

Just why there should be a smaller number of chromosomes in Pearlneck, in relation to Ring dove, which carry genes with species-specific effects on the serum, as com-

pared with the number whose genes affect the specific cellular antigens, is not at present answerable. One explanation might well be that many of the antigenic components of the cells are other than proteins (*i.e.*, they may be substances with antigenic activity because of linkage to proteins, *i.e.*, haptens), whereas those of the serum are probably proteins. On this basis, it would appear that the proteins are somewhat the more conservative genetic characters. But Pearlneck differs from Senegal in three or four cellular components and in about the same number of serum antigens, so that if there is a difference in the kind of antigens of serum and cells, those of the serum need not always be the more conservative.

A summary of all the comparisons which have been made between the serums of various species of animals, and between extracts or definite proteins of species of plants, would undoubtedly show that serum differences, presumably protein in nature, are one of the decisive tests in the differentiation of species. In other words, it is probable that all or nearly all species have protein specificity. Are protein differences, then, a line of demarcation only between species, or may there be such differences between individuals within a species?

Some months ago it was noted in our laboratory that the serums of hybrids between the pigeon (*Columba livia*) and Ring dove did not react alike following their use in absorptions of pigeon antiserum (unpublished data). Further tests lead to the conclusion that there were definite antigenic differences between the serums of individual hybrids, implying heterozygosity of the causative genes in the parent pigeons. If this be the correct explanation of the findings and their implications, differences definitely are to be found among the serums of individuals of this species. The small amount of serum that can be obtained from a single pigeon, however, almost precludes extensive tests of the correctness of this conclusion.

Following these findings, attempts were made to determine if there were individual differences in human serum. Definite evidence of such differences has been obtained very recently (Cumley and Irwin, 1943) in that the serums of some humans proved to be distinguishable from those of others. At the present writing, it must be admitted that there is a possibility that the antigenic substances in the serum were metabolic products of one kind or another, although it is much more probable that they represent definitive antigens. Should future tests establish that there are antigens in human serum which obey Mendelian principles, a new class of substances would become available for research in human genetics.

If these differences are genetic, and especially if it should be proved that they represent differences in proteins between individuals *within* a species—whether in humans or any other species—it obviously would no longer be correct to state that protein differences exist only *between* species. Furthermore, it appears reasonable to propose that any changes in the chromosomal material which affect the constitution of the proteins of a species would be a probable point of departure of incipient species. In any event, studies of the nature of the changes in chromosomal material required to affect the constitution of the proteins would be both interesting and valuable.

Because serum is made up of different kinds of proteins, one of the lines of work for the future is to determine whether the differences in serum antigens between species and between individuals are proteins, and what kind of proteins, or simply components linked to proteins. The immunological specificities of the types of pneumococci presented a problem to the biochemist, which, although brilliantly attacked, is still not completely solved. (Parallel studies made by other workers with various species of bacteria have put new life into research in bacteriology.) So also do the antigens of the cells and serum (and various body tissues as well), whether inter-

or intra-specific, challenge the biochemist for the analyses of the nature of the components. What is required for the future are the combined efforts of three branches of science—chemistry, genetics and immunology—each being dependent upon the other two for what humans are pleased to call the "final answers."

In conclusion, perhaps a bit of speculation on another subject may be permitted. Only within the last two decades have experimental findings allowed the immunologists to break away from the belief that only proteins were concerned in immunological reactions. By an ingenious technic, to which again we are indebted to Landsteiner, it has been demonstrated that relatively simple chemical substances, if attached to proteins, may be antigenic. Probably most geneticists at the moment are unable to think in terms of the constitution of genes as being other than protein. Leaning heavily upon findings in the field of immunochemistry, the suggestion is made that many genes may be less complex in nature than proteins—such as carbohydrates—but may owe their biological activities to their linkage to proteins. (It is probable that other biologists have somewhat the same concept of the possible nature of genes. For example, one of us in conversation with Professor C. E. Allen found that the above suggestion coincided very closely with one which he had held.) If the cellular antigens are more or less direct products of their causative genes, a chemical analysis of the nature of these antigens should be one of the most promising approaches to a knowledge of the chemistry of the gene itself.

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SEROLOGY AND ANIMAL SYSTEMATICS[1]

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INTRODUCTION

DISCOVERED by Rudolf Kraus in 1897, the precipitin reaction was first extensively applied to the problems of animal systematics by Nuttall, whose pioneer work in systematic serology is known, but not too well known, to most zoologists. Kraus (1897) at first understood the reaction to be absolutely specific, *i.e.*, each antiserum was capable of reacting only with the particular antigens used in its formation. Obviously such specific antisera can be used for *differentiating* antigens or the species characterized by them, but not for *classifying* them. So convinced were the early immunologists that the precipitin reaction was absolutely specific and therefore a grand device for identifying antigens, such as those present in blood stains, that Tchistovitch (1899) even claimed that antihorse serum would not react with ass serum, two closely related species being represented in these tests. Later investigators have had more difficulty in distinguishing between them, a distinction which can be made if the technique is more quantitative than that used by Tchistovitch.

The first report proving that the precipitin reaction was not absolutely specific was that of Bordet (1899) and the entire series of applications of the precipitin reaction to animal systematics rests on his discovery that an anti-chicken serum produced in a rabbit reacted most strongly with chicken serum *but also reacted, though much less strongly, with pigeon serum!* This was indeed the origin of the principle of quantitative specificity which now con-

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stitutes an essential part of the foundations of systematic serology. This principle states that each antiserum reacts most strongly with the particular antigens used in its formation (homologous reactions) and less strongly with other antigens (heterologous reactions) under comparable conditions. It was Nuttall's great contribution to have demonstrated the truth of this principle in the field of animal systematics and to have shown furthermore that the relative intensities of precipitin reactions did parallel the systematic positions of the species whose antigens were tested.

As a matter of fact Nuttall's first concern with the precipitin reaction was with its medico-legal application to the problem of identifying blood stains, but he quickly saw its possibilities as an aid to taxonomy. Thus he reported (1901a) on the results of testing several mammalian antisera, some of which showed weak cross reactions, and concluded: We have in this test the most delicate means hitherto discovered of detecting and differentiating bloods, and consequently we may hope that it will be put to forensic use."

But his next report (1901b), giving the results of testing 140 bloods and stating his intention of collecting and testing as wide a variety of bloods as possible, led him to say, "It seems certain that interesting results from the point of view of zoological classification will thus be brought to light." In fact, with each succeeding report his enthusiasm and faith in the precipitin reaction increased and his statements became more positive. From his next report (1901c) we quote: "The above experiments, which are being prosecuted on a large scale, the attempt being made to obtain a variety of antisera, indicate with certainty that we possess in this test a most valuable aid in the study of classification of animals."

In a subsequent progress report (1902) Nuttall described an improved technique of measuring the amounts of precipitate formed, gave some actual measurements which accord remarkably well with the systematic posi-

tions of the species tested, and made the following most interesting and understanding statements: "I do not wish these numbers to be taken as final, nevertheless they show the essential correctness of the previous crude results. To obtain a constant it will be necessary to make repeated tests with the bloods of each species and with different antiserums of one kind, making the tests with different dilutions and different proportions of antiserum. I am inclined to believe that with care we shall perhaps be able to 'measure species' by this method, for it appears from the above results that there are measurable differences in the reactions obtained with related bloods, in other words, determinable degrees of blood relationship which we may be able to formulate."

This is a most remarkable statement to have been made over forty years ago, showing that Nuttall had a basically correct understanding of the possibilities, both practical and theoretical, of quantitative precipitin testing. To "measure species" would indeed be a contribution to animal systematics of the greatest value, for taxonomy like all other branches of zoology must become more objective and quantitative to progress. But unfortunately Nuttall did not practise what he preached regarding the use of varying proportions of antisera and antigens and consequently he could not measure species—no, not even did he correctly measure the relative intensities of the reactions he performed. In spite of his 16,000 tests and more, summarized in his classic of 1904, he failed to use anywhere an adequate or good precipitin technique. In systematic serology useful ends can not be obtained without adequate means and to understand the sources of error in Nuttall's comparisons as well as those of many later investigators we must now turn briefly to matters of technique.

Some recent publications (Boyden, 1942; Boyden and De Falco, 1943, in press) have described the principal techniques used in precipitin testing as applied to animal systematics, and pointed out their chief sources of error.

The following account is, therefore, very brief. The two principal techniques used in precipitin testing and animal systematics are (1) the flocculation test and (2) the ring test. The former usually involves the mixture of constant amounts of antiserum with decreasing amounts of antigen, followed by the incubation of the reagents and the recording of the highest dilution of antigen in which appears a settled precipitate, or a greater turbidity than shown by the control tubes. Usually only the endpoint or "titer" is recorded, and the actual amounts of precipitate are ignored. Nuttall, however, used only a single, and *unknown,* amount of antigen, and crudely estimated, or later "carefully measured" the volume of precipitate obtained from that proportion of antigen and antibody. I say Nuttall used an unknown amount of antigen because the choice of a one to one-hundred or one to two-hundred dilution of any blood serum or filter paper extract of dried blood is really in a quantitative sense unknown as to its antigen concentration. But the flocculation test can be quantitatively performed with known amounts of antigen and antiserum, where the volumes of precipitate are measured after centrifuging (Boyden and Baier, 1929; Baier, 1933); where the nitrogen contents of washed precipitates are determined (Wu *et al.*, 1928; Heidelberger and Kendall, 1929); or where the turbidities developed may be accurately measured by such an instrument as the photronreflectometer (Libby, 1938). These are truly quantitative methods and are therefore superior to the earlier flocculation techniques. The ring test first developed by Ascoli (1902) involves a careful layering of the antiserum under successive dilutions of the antigen and it is usually recorded in terms of the titer or endpoint, *i.e.*, the highest dilution of antigen which shows a distinct layer of precipitate at the zone of contact of antigen and antiserum. Ring test endpoints should be sharper than the corresponding flocculation end points, and in this respect the ring test is superior to the crude flocculation test.

These essential matters of technique which must be understood before we may successfully evaluate the results of precipitin testing in the field of animal systematics may best be explained by reference to Fig. 1. Here are shown the data obtained from ring tests, and from photron-'er measurements of the turbidities resulting

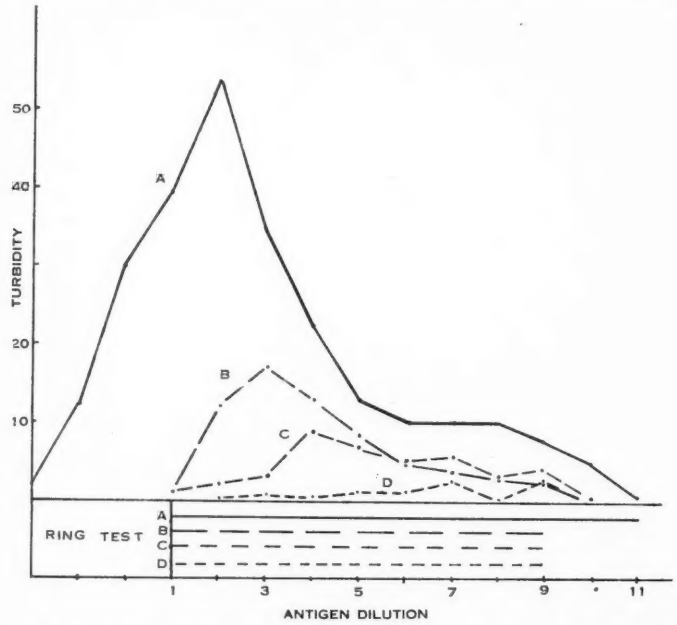

FIG. 1. A graph to show the results of testing an anti-*Callinectes sapidus* serum (I40b) with standard dilutions of the sera of *Callinectes sapidus* (line A), *Carcinus maenas* (line B), *Cancer borealis* (line C), and *Menippe mercenaria* (line D). The upper part of the figure gives the photron'er turbidities along the ordinate correlated with the antigen dilutions along the abscissa. Dilution "1" is one part of haemocyanin in 500 parts of buffered saline and each succeeding dilution has half the concentration of its predecessor.

The lower part of the figure shows the corresponding ring test titers for the same species and antigen dilutions.

from the interaction of an anti-*Callinectes sapidus* antiserum obtained from a rabbit, tested with comparable amounts of the sera of *Callinectes sapidus* (A), *Carcinus maenas* (B), *Cancer borealis* (C) and *Menippe mer-*

cenaria (D). The lower part of the figure gives the ring test results; the curves above are the turbidities as measured by the photron'er. Galvanometer readings of the photron'er appear on the ordinates, the antigen dilutions are plotted along the abscissa. Dilution "1" represents one part of haemocyanin to 500 parts of buffered saline and each succeeding dilution is one half the concentration of its predecessor. Thus dilution "2" is 1 : 1000 and "3" is 1 : 2000, etc.

Now the salient points obtainable from a study of Fig. 1 are these:

(1) The homologous reactions exceed all others to a considerable degree.

(2) The relative intensities of the reaction accord well with the systematic positions of the species.

(3) The photron'er comparisons give readings throughout the entire reaction range of each test whereas,

(4) The ring test records only the endpoints or titers and is therefore generally a less adequate and less discriminating method for the comparison of related bloods.

(5) The photron'er tests show the real nature of the precipitin reaction as a phenomenon of optimal proportions, and compare the antigens throughout their entire reaction range with a given antiserum, whereas the ring test records only the titer or relative sensitivity of an antiserum when tested with different antigens.

Now this comparison of a truly quantitative flocculation technique with the widely used ring test gives all the advantages to the former. Actually there is one more respect in which the photron'er comparisons surpass the ring test, *viz.*, the former *requires no independent proof of equivalence in the amounts of antigen used* provided the curves are complete, touching or nearly approaching the value of zero turbidity at each end. For ring test titers to be comparable, when an antiserum is tested with a variety of antigens, the antigens compared must be tested in equivalent amounts, for the position of the endpoints is dependent on the relative sensitivities of an antiserum *measured by* the smallest concentrations of the various antigens capable of reacting with it. If these concentrations of antigen are unknown or not equivalent the whole basis of comparison becomes invalid.

On the other hand, provided the curves are complete, the photron'er gives the relative reaction intensities of an antiserum with a variety of antigens and no more reaction is possible. In view of the fact that serological equivalence is usually difficult to establish, it is obvious that the photron'er's inherent advantages justify our inclusion of no further ring test results in this report.

One more consideration requires further explanation. The fact that the precipitin reaction is a phenomenon of optimal proportions, so nicely illustrated in Fig. 1, makes it obvious at once that the proper basis of comparison in quantitative flocculation tests is the curve, the whole curve and nothing but the curve. It is clear that any lesser basis of comparison could fall into serious error. Thus there is no single antigen dilution which falls on the same relative position to the whole curve for the different sera tested. Therefore, since the whole reaction range of each antiserum and each antigen must be determined, the natural procedure which we have adopted is to compare the relative areas of whole curves. No other basis for comparison is adequate, and here is where Nuttall made a great mistake. He "carefully measured" the volume of precipitate at some one unknown point of the curve of optimal proportions and thus he and others too have given us data of unknown and unproven comparability. Far from "measuring species" they failed to adequately measure the relative intensities of the precipitin tests made by them.

With this brief discussion of essentials regarding technique we should be able to properly evaluate the data already obtained and those presented in this and subsequent reports. But now what of the general theory of systematic serology? Where does systematic serology stand in relation to zoology as a whole? The fundamental principles of systematic serology are these:

(1) The antigenic composition of animals is an important part of their essential natures and must be considered in any sound natural system of classification.

(2) Protein antigens are conservative hereditary traits.

(3) Good precipitin techniques are well adapted to reveal the relative degrees of biochemical similarity of protein antigens.

With regard to these principles the first can scarcely be disputed. It is generally admitted by taxonomists that the more we know of animal nature, the more "natural" our classifications may become. From the standpoint of objectivity alone, biochemical comparisons outrank most morphological description. Paleontologists, from obvious limitations, may be restricted to morphological comparisons and fragmentary ones at that, but surely no one will recommend that taxonomists ignore the biochemical composition of recent organisms which is as essential a part of their natures as any morphological features. Actually biochemical comparisons fall within the province of "morphology," though in this case it is a matter of the structure and configuration of atoms and molecules.

The second principle is a short statement of the essential truth that proteins are at least as constant and characteristic of animal species as their morphological features and are fundamentally determined in their expression by a genetic mechanism. It is true that the specific nature of an organism is determined primarily by inheritance and this inheritance applies to proteins as well as to gross morphological features. Recently the qualities of serum proteins have been found to be inherited. Thus Boyden (1942 and earlier) finds that the serum of the mule is intermediate between that of horse and ass, and Cumley, Irwin and Cole (1941), and Cumley and Irwin (1942) report the typical inheritance of serum protein differences and resemblances in their dove hybrids. So far as is known at present the quality of the serum proteins is not affected by changes in the environment and thus serum protein antigens bear the characteristics of conservative inherited traits.

Finally, the relative intensity of precipitin reactions, when adequate techniques are employed, is a measure

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Finally, the relative intensity of precipitin reactions, when adequate techniques are employed, is a measure

of the serological and chemical similarity of the antigens tested and this principle has been abundantly proved by the investigations of Landsteiner and his associates as summarized in Landsteiner (1936) and by the investigations of Marrack and others reviewed in Marrack (1938). Some of the comparisons between antigenic constitution and serologic specificity reported by these workers were based on relatively crude techniques and further studies of a more truly quantitative nature are needed but there is no reason to doubt the general proportionality between chemical nature and serologic specificity.

The theory of systematic serology is sound, but there have been too few adequate applications of good precipitin techniques to problems of animal systematics. We are at present far from the goal of systematic serology but the potentialities for useful contributions to the solution of taxonomic problems have become clearer in recent years. As an example of such potentialities we present briefly the results of recent studies on Crustacean relationships as obtained by the new photron'er technique. These will be briefly compared with the results of photron'er studies on other groups of animals, which have been conducted in our laboratory.

PHOTRON'ER COMPARISONS OF THE SERA OF COMMON CRUSTACEA AND THEIR INTERPRETATION

These studies came chiefly as a result of the interest, aid and encouragement of Dr. Waldo L. Schmitt, of the U. S. National Museum. It is a pleasure to acknowledge my debt to him for the loan and gift of specimens, for aid in their collection, for the identification of species and for advice regarding the problems of Crustacean systematics. I am indebted also to Dr. A. C. Redfield, of Harvard University, for first pointing out the fact that Crustacea are favorable species for serological studies on account of the high proportion of a single protein, haemocyanin, in their sera. Actually, according to Allison and Cole (1940), haemocyanin is the only protein present in the sera of common Crustacea.

The antigens were collected over a period of years involving four trips to the Tortugas Laboratory of the Carnegie Institution, and one trip to the United States Bureau of Fisheries Laboratory at Beaufort, N. C., one to the Mt. Desert Island Biological Laboratory at Salsbury Cove, Me., and one trip to the Laboratory of the Marine Biological Association at Plymouth, England. The blood was obtained from the various species of crabs by the removal of a fifth pereiopod, and from the lobsters and crawfish by a ventral incision through the membranes in the region of junction of cephalothorax and abdomen. For large animals the blood from single specimens was sometimes kept as separate samples; in all other cases pooled bloods were obtained. These blood samples were allowed to clot, stored for a few hours in the refrigerator, and the serum was poured off. These sera usually remained in an oxidized state and were so filtered through Seitz filters and stored sterile in small vials. They have been kept in the icebox since their arrival in the Rutgers laboratory.

The antisera were, in most cases, prepared in rabbits in accordance with a standard injection procedure, *i.e.*, the initial intravenous dose was 5 mgms of protein per kg of body weight of the rabbit, followed by three other doses on alternate days, each of which contained twice the protein content of the preceding dose. Seven to ten days after the last injection the rabbits were bled completely, under ether anesthesia, by cardiac puncture. The rabbit's blood was allowed to clot with minimum disturbance, and the serum was collected as formed. The antisera so obtained were filtered sterile through Seitz filters and stored in small vials in the refrigerator.

The photron'er tests were performed as briefly described by Boyden (1942) and as described in detail by Boyden and De Falco (1943, in press). The data obtained as a result of testing fourteen antisera with a variety of Crustacean sera are shown in Figs. 1, 2 and 3 and summarized in Tables I and II.

Fig. 1 (upper part) shows the results of testing an antiblue crab serum (I40b) with the sera of the blue crab, *Callinectes sapidus* (line A); the green crab, *Carcinus maenas* (line B); the Jonah crab, *Cancer borealis* (line C), and the stone crab, *Menippe mercenaria* (line D). The relative areas under the curves are as follows: A,

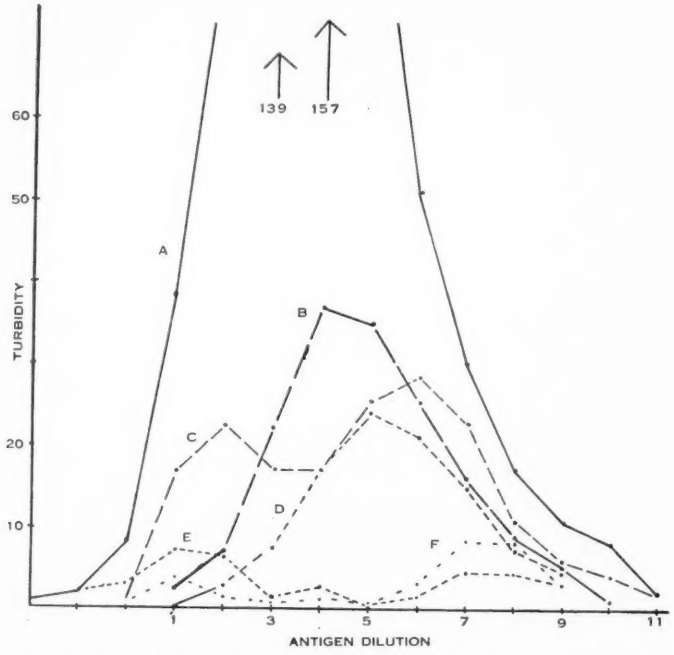

FIG. 2. A graph to show the turbidities resulting from the titration of a powerful anti-Menippe serum (I63) tested against the following antigens: *Menippe mercenaria* (A), *Geryon quinquedens* (B), *Callinectes sapidus* (C), *Cancer borealis* (D), *Ocypode albicans* (E), and *Maia squinado* (F). The corresponding areas are given in Table 1. This antiserum as diluted 1 plus 2 parts of saline gave a homologous area of 645 units. It could have been further diluted but was used as the 1 plus 2 dilution in order to obtain significant measurements of the more distantly related families.

homologous reaction, 100 per cent.; B, 26 per cent.; C, 16 per cent. and D, 4 per cent.

Fig. 1 (lower part) shows the results of the ring tests on the same species. Here the corresponding titers are

100 per cent., 25 per cent., 25 per cent. and 25 per cent., respectively.

Obviously the differentiation of the species tested is better shown by the photron'er results than by the ring test results. The previous discussion of the two techniques should explain why the photron'er is capable of more adequate comparisons than the ring test. Furthermore, the photron'er data for these species accord pretty well with their systematic positions whereas the ring test results do not. Thus Callinectes and Carcinus are genera of one family, the Portunidae; whereas Cancer and Menippe belong to the different families, Cancridae and Xanthidae, respectively.

Fig. 2 shows the reactions of a more powerful antiserum (163—anti-*Menippe mercenaria*). It was tested with the sera of Menippe mercenaria (A); *Geryon quinquedens* (B); *Callinectes sapidus* (C); *Cancer borealis* (D); *Ocypode albicans* (E), and *Maia squinado* (F). The range of this antiserum was great enough to include all the families of crabs tested; indeed, from the mountain peak of 157 units turbidity, one should be able to see even as far as the spider crab, Maia, way over on the horizon. As far as can be stated at present, the relative curve areas constitute a fair approximation to the systematic positions of these species, though powerful antisera are generally less specific than antisera of moderate strength and the area of the Callinectes curve is too large in comparison with the reciprocal tests given in Table I. We have shown (Boyden and De Falco, 1943, in press) how powerful antisera may be made more specific and more discriminating by dilution, and thus how antisera of different original grades of specificity may be standardized.

The curves shown in Figs. 1 and 2 are typical of a large number obtained in the study of other animal groups besides the Crustacea such as Insecta, Pisces, Aves and Mammalia. Occasionally a bimodal curve is obtained or the curve may run a more rounded course, but these differences do not appear to justify the presentation of

more curves at this time. Instead the data from all the Crustacean comparisons are summarized in the tables which follow. In all cases the homologous area represents 100 per cent. and the heterologous per cent. values indicate the ratio of heterologous areas to the homologous area.

The data shown in Table I are incomplete since not all the antisera have been tested with a sufficient number of

TABLE I

A COMPARISON OF THE SEROLOGICAL REACTIONS OF THE SERA OF COMMON CRUSTACEA

Anti-serum	Homologous antigen	Test antigens	Homarus americanus	Homarus vulgaris	Callinectes sapidus	Carcinus maenas	Cancer borealis	Cancer irroratus	Cancer pagurus	Menippe mercenaria	Geryon quinquedens	Ocypode albicans	Mala squinado
I42(1+2)	Homarus americanus	L3	*100*	54									
I40(1+0)	Callinectes sapidus	L4			*100*	26	16						
I47(1+0)	Callinectes sapidus	371A			*100*	44	14						
I54(1+1)	Callinectes sapidus	HC38–1			*100*	17	17			7	3		1
I52(1+0)	Carcinus maenas	3			34	*100*	22			8			1
I48(1+1)	Cancer borealis	3b					*100*	58					
I51(1+1)	Cancer borealis	HC1					*100*	59	41				
I60(1+1)	Cancer borealis	3d					*100*	29	19				
I61(1+1)	Cancer pagurus	39–1					41	33	*100*				
I62(1+0)	Cancer pagurus	39–2					55	51	*100*				
I49(1+0)	Menippe mercenaria	36–A			6		12			*100*	10		
I63(1+2)	Menippe mercenaria	36–A			28		16			*100*	25	6	5
I50(1+1)	Geryon quinquedens	36–1						19		24	*100*		
I64(1+1)	Geryon quinquedens	39–2			3					6	*100*		

antigens. From the standpoint of animal systematics, however, they appear to have a special interest and since it may not be possible to complete these tests in the near future they are reported at the present time.

From the standpoint of animal systematics the data of Table I may best be presented as outlined below, for here the values are assembled so as to show most clearly their relation to the systematic categories concerned.

I. The relationships of the sera of species of the same genus.
  A. Cancer
    1. *Cancer borealis vs. C. pagurus* 41, 41, 19, 55 av. *39*
    2. *Cancer borealis vs. C. irroratus* 58, 59, 29 av. *49*
    3. *C. pagurus vs. C. irroratus* 33, 51 av. *42*
  B. Homarus
    1. *H. americanus vs. H. vulgaris* 54 *54*
    Grand average *46*

II. The relationship of the sera of genera of the same family.
  A. Callinectes vs. Carcinus 26, 44, 17, 34 av. *30*

III. The relationship of the sera of different families of Brachyura.
  A. Portunidae vs. Cancridae 16, 14, 22, 13, 17 *av. 16*
  B. Portunidae vs. Xanthidae 4, 6, 8, 7, 6 (28) average of 5 values *6*
  C. Portunidae vs. Goneplacidae 3, 3, *av. 3*
  D. Portunidae vs. Maiidae 1, 1, *av. 1*
  E. Cancridae vs. Xanthidae 12, 16, *av. 14*
  F. Cancridae vs. Goneplacidae 24, 4, *av. 14*
  G. Xanthidae vs. Goneplacidae 10, 25, 24, 6, *av. 16*
  H. Xanthidae vs. Ocypodidae *6*
  I. Xanthidae vs. Maiidae *5*

Now the values thus outlined must be considered tentative, for we have not yet reached the plane of attack on these problems at which we can guarantee the serological equivalence of the antisera and antigens tested. The variables and sources of error are discussed below. The data do give us a first quantitative approximation to the serological relationship of the species tested and they do appear generally to agree with their systematic positions. Of special interest in this connection may be the relatively sharp differentiation between the sera of *Homarus americanus* and *H. vulgaris*. These species are very similar morphologically and might even be mistaken for one species if their geographic distribution were continuous, but their sera are almost as different as those of the three species of Cancer are to each other. The conclusion appears to be justified that *H. americanus* and *H. vulgaris* are "good" species, and that they would probably not be able to hybridize even if their ranges were continuous. Another case of special interest may be the position of Geryon, representing the family Goneplacidae, in relation to the families Xanthidae, Cancridae and Portunidae.

According to Miss Rathbun (1918, p. 9) the family Goneplacidae "is most closely allied to the family Xanthidae" and our data confirm this conclusion but show in addition that the Goneplacidae are almost as close to the Cancridae as to the Xanthidae. More tests with a variety of antisera and antigens are needed in order to obtain a "constant," as Nuttall termed it, but the position of Geryon and the Goneplacidae in relation to the other families as

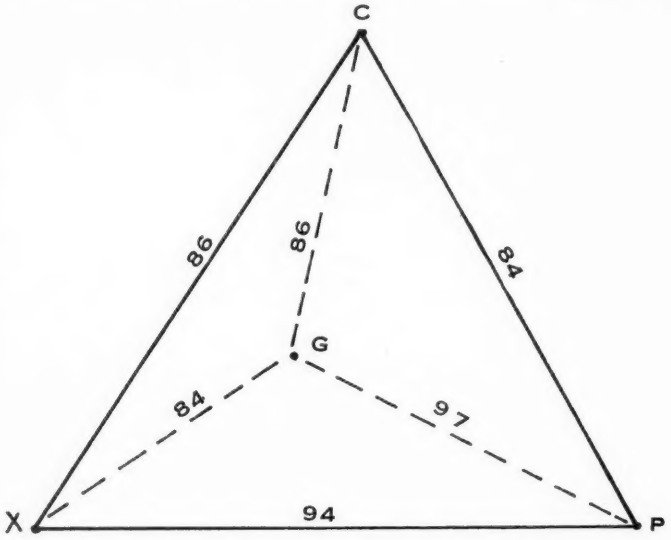

FIG. 3. A diagram to show the relative distances of 4 families of Crustacea from each other. The data are tentative inasmuch as the families have not yet had really adequate testing. The families concerned are: Cancridae (C), Portunidae (P), Xanthidae (X), and Goneplacidae (G). The latter family represented by Geryon is apparently closest to the Xanthidae represented by Menippe, a little less close to the Cancridae and considerably more distant from the Portunidae. Three dimensions would be required to express these relationships properly and the plane figure is really a projection of such three-dimensional distances onto a plane surface.

indicated by the present data is shown in Fig. 3. Actually three dimensions would be needed to express this relationship properly, but it can be done on a plane surface as shown here.

It must be understood that further tests are indicated not only to discover the normal values for these species but to gain a fairer representation of the families concerned. Thus Menippe has had to serve as the sole representative of the Xanthidae, and Geryon as the sole representative of the Goneplacidae. We hope later to have filled some of these gaps in the serological analysis of Crustacean systematics.

Meanwhile the variables, the sources of error, in the photron'er technique, must be continuously attacked. The photron'er appears to have solved that part of the problem of antigenic equivalence which is concerned with

TABLE II

THE EFFECTS OF INACTIVATION AND OF THE ADDITION OF COMPLEMENT

Antiserum condition	Test antigen	Condition	Curve area	Per cent.
I60(1+0) native	*Cancer borealis* 3b	native	289	100
" "	" "	inactive	164	57
I60(4+1 saline) native	*Cancer borealis* 1b	native	234	100
" "	" "	inactive	151	65
" "	*Cancer borealis* 2b	native	249	100
" "	" "	inactive	120	48
" "	*Cancer borealis* 3b	native	179	100
" "	" "	inactive	102	57
" inactive	" "	inactive	25	14
I60(4+0) inactive then add 1 part of complement	" "	inactive	67	37

the *amounts* of antigen to be used in the tests. As long as the curves are complete, the antigens are present in comparable amounts. But the photron'er has not as yet solved the problem of the *quality* of the antigens tested. The fact is that different samples of the same species of antigens may show different amounts of reactivity with the same antiserum. This is true even with pooled sera. We have already reported that mammalian sera show a slow decrease in reactivity over a period of years even though kept in the icebox. Recently we have discovered that complement may be concerned in precipitin reactions contrary to the current understanding among immunologists. The results of tests involving the inactivation of the reagents and the addition of complement are shown in Table II.

We note from Table II that inactivation of the antigen for a half hour at 56° C. cuts down the turbidity to an average of 57 per cent. of its original value and that when both antigen and antiserum are inactivated the value was lowered to 14 per cent. Addition of complement, presumably in excess, brought about only a partial restoration of the reactivity. The general understanding among immunologists that inactivation has no effect on precipitin reactions is probably due to the crudity of techniques previously employed and should be corrected.

Other variables involved in the measurements given include the degree of specificity of the antisera. It was discovered long ago and has been abundantly confirmed by Wolfe (1939 and earlier) that prolonged immunization leads to the production of less discriminating antisera of broad range, whereas a short injection procedure involving small amounts of antigen leads to more discriminating antisera of narrower range. Obviously antisera of the same grade of specificity must be selected if comparable results are to be gained. The antisera used in this report were not standardized as to their specificity though they were produced in a comparable manner. In the future we hope to use standard antisera, selected with regard to their grade of specificity as demonstrated by their reaction with some particular homologous and heterologous antigens.

It is known also that lipoids may be concerned in precipitin reactions. Thus it was reported (Boyden, 1936) that convergent ring test results were obtained in Crustacea, results which could be corrected by the removal of ether-soluble substances from the antigens. On the other hand, the photron'er comparisons with native sera appear to show little if any disturbance of this sort.

When these and other sources of error in the photron'er method are corrected we should be able to use serologically equivalent antigens and antisera. Even at present we can see the trend of the results and gain our first approximations to the quantitative measurement of the relationships of animals as indicated by their sera.

There appears to be only one other recent study of Crustacean relationships involving precipitin reactions. A report by Clark and Burnet (1942) contains some interesting and valuable data regarding precipitin tests on Australian Crustacea. Using the ring test or a crude flocculation test involving no measurements they find a general parallelism between the amounts of precipitate formed and the systematic positions of the species tested. They call attention to the relatively narrow range of their antisera, apparently not realizing that the inactivation of all their antigens and antisera must have considerably cut down the reactivity of their reagents. Absorption experiments were used to differentiate closely related species. Tests with the purified haemocyanin of one species disclosed the haemocyanin as the principal if not the only antigen contained in that serum. This result accords with the analyses of Allison and Cole on other species of Crustacea. The data thus indicate that with a more quantitative technique consistent measurements of the serum relationships of these species could be obtained. From the standpoint of technique [illegible] tests on Crustacean sera performed by von Dungern (1903), Nuttall and Graham-Smith (1904) and Erhardt (1929) need not be examined at length.

Serological Measurements in Relation to Systematic Categories

To date the photron'er has been, or is being, applied to the study of Mollusca (Chestnut, unpub.) Crustacea, Insecta (Leone, unpub.) Pisces (Gemeroy, in press), Aves (De Falco) and Mammalia (Boyden, 1942 and unpub.). Essentially the same types of results have been obtained in all these groups, and no one by looking at a series of curves could tell from which group of animals the curves were derived. More than that the amounts of serological divergence run, in general, in accordance with the rank of the systematic category, even though in not a single one of these studies was any attempt made to select antisera of certain grades of specificity. Thus to take data from

the comparison of common Mammalia we find the following values for their serological resemblances:

I. For members of the same genus
   Horse *vs.* ass, 85 per cent. (Boyden, unpub.).
II. For related genera of the same family:
   Brook trout *vs.* Rainbow trout, 66 per cent.
   Brook trout *vs.* Brown trout, 53 per cent. } (Gemeroy, in press)
   Black bass *vs.* Large mouth bass, 75 per cent.
   Bison vs. cow, 73 per cent. (Boyden and De Falco, in press)
III. For closely related families:
   Bovidae *vs.* Cervidae, 45 per cent. (Boyden, unpub.)
IV. For distantly related families:
   Bovidae *vs.* Suidae, 5 per cent.

In Aves (De Falco, 1942) the values are generally higher in all the distantly related species, for ordinary antisera can apparently react with all the orders of birds suggesting that the amount of serological differentiation between orders of birds is roughly comparable to that between the more distantly related families of mammals.

The conclusion from this brief consideration of "serological dimensions" appears to be justified that precipitating antisera produced in rabbits may be used as a kind of taxonomic "yardstick" for measuring the magnitude and differentiation of systematic categories. It may even be possible with standardized antisera to set up limits for defining systematic categories and thus aid in the eternal debate between the "splitters" and the "lumpers." At any rate, the photron'er has thus far given some rather promising data, indicating that it may be useful in the arrangement of species within a genus, of related genera and of related families. In birds it may also aid in the difficult taxonomic problem of arranging the orders in accordance with a natural system.

The fact that the serological measurements fall off with the more distant systematic categories in a fairly regular way may also have a bearing on the nature of the evolutionary processes which have produced the various types of organisms in these categories. It has been ably maintained by Mayr (1942) that the evolution of subspecific and superspecific types is essentially the same type of process. On the contrary, Goldschmidt (1940) claims

that there is a difference in kind and in mechanism between these two grades of evolutionary divergence. So far as the serological tests discussed in this report go, they appear to confirm Mayr, for they indicate an apparently continuous evolutionary differentiation of the serum proteins. Thus individual serological variation grades into specific and specific into generic differentiation, with the amount of difference a matter of degree rather than of kind for all the systematic categories.

These general conclusions are supported by the recent valuable contributions of Cumley (1940), of Wilhelmi (1940, 1942) and of Wolfe (1939b). A recent review (Boyden, 1942) discusses these and other results in some detail, presents a critique of the principles of systematic zoology and discusses the relation of systematics to general biology from the point of view of a serological attack on these problems.

It is a marvelous thing that any animal, even such as the rabbit, should possess the capacity to produce, under appropriate conditions, antisera of innumerable kinds, each capable of reacting with a variety of chemically related substances in accordance with their chemical and serological resemblances. So far, the rabbit has actually made fewer mistakes than man in the attempt to construct a natural system of classification, for the serological data derived from the recent and more effective use of rabbit antisera have given a more consistent basis for the determination of animal relationship than has previously been developed on other grounds by men whose views regarding some of the more distant systematic relationships have been and still are widely divergent.

Summary

The photron'er method has now been applied to the study of systematic relationships in representative Crustacea, Insecta, Pisces, Aves and Mammalia. This method of serological comparison has definite advantages over previous methods of study; in particular, it has solved the problem of the serological equivalence of antigens as far

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as the *amount* of such antigens is concerned. The data reported as a result of these studies indicate that the photron'er technique may be usefully applied to the comparison of the species of a genus, the genera of a family and to the families of an order. In birds the method may also be used in the comparison of representatives of related orders. The data from these various studies have led to a first quantitative approximation of the amounts of serological divergence characteristic of systematic categories of different rank and indicate that such data may provide a kind of serological "yardstick" for the delimitation of systematic categories. Further improvements in technique and a wider application of the method are needed if we are to make rapid progress in the application of serological techniques to animal systematics.

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QUANTITATIVE GENIC–HORMONE INTERACTIONS IN THE FOWL

I. RELATIVE SENSITIVITY OF FIVE BREEDS TO AN ANTERIOR PITUITARY EXTRACT POSSESSING BOTH THYROTROPIC AND GONADOTROPIC PROPERTIES

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IN previous publications from this laboratory (Munro, 1936; Munro, Bird and Hopkins, 1937) it has been suggested not only that the number of eggs produced by domestic fowl during their first laying year is very largely determined by non-genetic factors but also that that portion of the variance caused by genetic factors (20–25 per cent.) is the result of the action of genes which could be divided roughly into two sorts. First, the "primary" egg genes which are concerned with fundamental physiological processes, *e.g.*, functional level of endocrine glands, sensitivity of soma to sex endocrines, efficiency of feed utilization, etc., and second, the "secondary" or "protective" genes which enable the organism to ward off disease or to function normally or more normally than individuals not possessing them, under such adverse conditions as deficient rations, extremes of temperature, etc. Since the environmental variations with which the protective genes interact are changing continuously, then egg production, in so far as it is influenced by these protective genes, will fluctuate in accordance with the interaction between the individual's complement of protective genes and the changing environment. On the other hand, it would seem that the primary egg genes should function more or less continuously under varying conditions but their actions, as measured by rate of egg production, would be, in most cases, almost completely masked by both purely environmental factors and the action of the protective genes.

It would appear that evidence for the existence of such primary genes might better be obtained by the use of other criteria than the egg production rate of adult females. For instance, if a breed or strain possesses a genetically determined low threshold of response to gonadotropic hormone, then administration of the hormone to responsive individuals of that breed should result in a greater physiological effect than similar administrations to a breed possessing a higher response threshold. If such breed differences could be proved, one would be justified in inferring that gonad development in some breeds require less of the native gonadotropin than others from which it would appear to follow that in these breeds egg production would be initiated at lower levels of pituitary activity.

There is considerable evidence showing that qualitative breed and varietal differences in feather morphology and coloration are controlled by intracellular (genic) rather than extracellular (humoral) factors (see review by Danforth, 1939). On the other hand, evidence on the relative influence of genic and humoral factors on quantitative physiological reactions is scanty. However, there is a growing list of evidence that such characters are also under genic rather than humoral control. Among these may be mentioned the differences between the comb of the Leghorn and the Plymouth Rock in degree of response to androsterone (Callow and Parkes, 1935); the findings of Bates, Riddle and Lahr (1939) with respect to differences between breeds of pigeons in the degree of crop-sac response to prolactin; the fact that animals from different colonies may vary widely in their sensitivity to gonadotropin (Barlow and Sprague, 1941). The report by Byerly and Burrows (1936) indicating that the pituitaries of broody breeds of chickens contain more prolactin than those of non-broody genetic constitution is one of the few indicating that functional physiological traits of a genetic nature are humorally controlled. In order to gain evidence for the existence of the postulated

primary egg genes as well as to add to existing knowledge concerning the control of physiological traits a series of studies was undertaken at this laboratory to determine the effect of the genotype on hormone reaction in the fowl. The present paper deals with breed differences in the response of certain primary and secondary sex organs to the injection of a pituitary extract containing both thyrotropic and gonadotropic fractions.

MATERIALS AND METHODS

Baby chicks were chosen in preference to older birds for a number of reasons: (1) They are easier and less expensive to produce in quantity; (2) they require less hormone, and (3) they should make better experimental material since the action of any extraneous hormones used would be interfered with little by endogenous hormones from their comparatively inactive sex organs. Five popular breeds, White Leghorn, New Hampshire, Light Sussex, White Plymouth Rock and White Wyandottes, were chosen. These are all single comb with the exception of White Wyandotte, which is a rose-comb breed. White Rocks were included because of the report of Nalbandov and Casida (1940) who found the immature testis of this breed to exceed the White Leghorn in sensitivity.

It is obviously impossible to obtain a representative cross-section of any one breed and still keep the number of individuals involved within the physical limits of an experiment of this sort. The chicks were obtained from one of the largest commercial hatcheries in Canada and they are therefore about as representative of their breed as it is possible to get without greatly increasing the numbers and adopting a method of restricted or stratified sampling. Upon arrival the chicks were placed in electrically heated brooders. The chicks were apportioned among five compartments in such a way that each compartment had a fairly even representation of each breed. This was done to equalize environmental conditions

among the breeds. A six-day period of observation was allowed to elapse before treatment began, during which time all unthrifty individuals were removed. A total of 533 healthy chicks remained and were used in this study.

Injections were started on the seventh day, continuing daily for ten days. Prior to the commencement of the treatment period, each breed-group was divided into an experimental and a control lot. Each of these lots consisted of males and females in approximately equal proportions. Breeds, sexes and treatments were re-allotted at random to the five compartments.

The pituitary preparation employed consisted of a pooled sample of two chemically extracted dry powder concentrates of glandular origin, A.P. 61 B and A.P. 81 B, which in previous work on chicks in this laboratory had been found to possess chiefly gonadotropic and thyrotropic properties, respectively. They were very kindly supplied to the senior author by Dr. A. S. Parkes, of the National Institute for Medical Research, London, England. The relative biological potency of these extracts were determined in Dr. Parkes' laboratory with rats or mice as the test animal. However, unpublished work in our laboratory on a variety of extracts from the same source has shown that this potency does not agree with that determined on chicks. The extracts used in the present study were among the more potent in so far as their effects on chicks are concerned. The treatment consisted of a daily injection of 1.83 mg of the pooled extract dissolved in 1 cc of a slightly acidified aqueous solution which was brought to the neutral point just before injection. The total amount of A.P. concentrate supplied to an individual chick during the ten-day injection period was slightly over 18 mg. Control chicks received the solvent only.

Twenty-four hours following the last injection, the chicks were weighed and then killed by gas. The combs, thyroids and gonads were removed in both sexes. In the females, the oviduct was also excised. Upon removal,

the organs were weighed on a chainomatic balance to the nearest milligram.

Because of the intimate relationship between body and organ weights (Kosin, 1940), the latter had to be adjusted to a uniform body weight. Although in an earlier paper (Munro and Kosin, 1940) organ weights were expressed as a per cent. of body size, in the present work a different, and what is believed to be a more critical basis for comparison was adopted.

Hopkins and Biely (1935) concluded that as the size of the bird increases, the average percentage of the total body weight due to liver, kidney or spleen decreased. Data collected in this laboratory show that this tendency for an organ weight to become relatively smaller (actually larger in absolute terms) as the body weight increases also holds true for most of the endocrine organs in young fowl. Thus, the larger birds would be automatically penalized in any experiment in which organ weights were being studied and more especially it would be impossible to make a valid comparison of organ weights between breeds differing in size.

The difficulty can be overcome by correcting the mean organ weights of the various groups to be compared to a common body weight by means of the regression equation which characterizes each group. This, in effect, provides a measure of organ weight which, in theory, would occur if all birds were equal in body weight. The variability between organ weights which still exists is a net variability freed from the influence of variation in body weight, and the corrected means for the various groups thus secured have correspondingly reduced standard errors. These reduced standard errors, technically known as standard errors of estimate, are used to calculate the significance of the differences between the corrected means in the usual way. This procedure can be found outlined in any modern statistical text and is lucidly explained by Wallace and Snedecor (1931). Table 1 illustrates the relative standing of the five breeds

used in this experiment when (a) the organ weights are corrected to a common body weight by the method of regression and (b) the organ weights are expressed as a per cent. of the body weight.

It can be seen that the relative order of sensitivity is not the same in the two methods. We consider the re-

TABLE 1

ORDER OF BREED SENSITIVITY TO A.P. EXTRACT ACCORDING TO TWO METHODS OF COMPARISON. (ROMAN NUMERALS = ORGAN WEIGHTS CORRECTED TO UNIFORM BODY WEIGHT BY REGRESSION. ARABIC FIGURES = ORGAN WEIGHTS EXPRESSED AS PERCENTAGE OF BODY WEIGHT)

Organ	Comb	Thyroid		Gonad
Sex	♂♂	♂♂	♀♀	♂♂
White Leghorn	I 1	III 5	III 2	III 4
White Wyandotte	IV 5	I 1	I 1	II 2
Light Sussex	V 4	II 2	IV 4	IV 3
New Hampshire	II 2	IV 4	II 3	V 5
White Rock	III 3	V 3	V 5	I 1

gression method to be the better one and it has been employed in analyzing the data presented in this paper.

RESULTS

One of the first things which emerged from this study is the fact that basic differences exist in the size of the same organs in the control birds of different breeds. At seventeen days of age, the White Leghorn chicks of both sexes had the heaviest combs, thyroids and gonads, while the females of this breed also had the heaviest oviducts (Table 2). Because of this fact it is debatable whether the measure of breed sensitivity should be the simple excess of injected over control organs or whether this excess should be expressed in terms of the control. For instance, the injected Leghorns showed an increase of 25 mg over the control in weight of comb, while the corresponding increase for New Hampshires was only 12 mg. However, the 12 mg increase shown by the New Hampshires represents an increase of 66.7 per cent. over the controls, whereas the 25 mg increase in the Leghorns is only 62.7 per cent. higher than the controls. The data are summarized in Table 2, and it will be seen that the

TABLE 2

A Summary of the Data Showing the Corrected Organ Weights for Each Breed and Sex Group. In Each Column the Breeds Are Arranged in Descending Order of Organ Size and in the Case of Injected Birds the Absolute and Percent Increase Over the Controls is Also Shown
W.L.—White Leghorn; N.H.—New Hampshire; L.S.—Light Sussex; W.R.—White Rock; W.W.—White Wyandotte

Comb				Thyroid				Gonad				Oviduct	
Male		Female		Male		Female		Male		Female		Female	
Injected	Controls	Injected	Controls	Injected	Controls	Injected	Controls	Injected	Controls	Injected	Controls	Injected	Controls
WL 65 ± 3.7 + 25 ± 5.16 + 62.5%	WL 40 ± 3.6	WL 33 ± 1.4 + 4 + 13.8%	WL 29 ± 1.6	WW 17.7 ± 1.3 + 7.0 ± 1.36 + 65.4%	WL 10.9 ± 0.8	WW 19.3 ± 1.3 + 8.5 ± 1.36 + 78.7%	WL 12.1 ± 0.7	WL 70 ± 2.9 + 32 ± 3.18 + 84.2%	WL 38 ± 1.3	NH 53 ± 2.8 + 22 ± 3.22 + 71.0%	WL 34 ± 2.1	WL 27 ± 1.1 + 2 + 8.0%	WL 25 ± 1.0
WW 39 ± 1.2 + 7 ± 2.08 + 21.9%	WW 32 ± 1.7	WW 28 ± 1.5 + 2 + 7.7%	WW 26 ± 1.9	LS 14.2 ± 0.6 + 3.9 ± 0.78 + 37.9%	WW 10.7 ± 0.4	WL 16.7 ± 0.9 + 4.6 ± 0.98 + 38.0%	LS 11.1 ± 0.4	WR 70 ± 5.2 + 42 ± 5.65 + 150.0%	NH 36 ± 2.6	WL 50 ± 2.2 + 16 ± 3.04 + 47.1%	NH 31 ± 1.6	WW 23 ± 0.9 + 1 + 4.6%	WR 23 ± 1.7
LS 32 ± 2.4 + 6 ± 2.78 + 45.5%	LS 26 ± 1.4	LS 22 ± 1.3 + 2 + 10.0%	NH 21 ± 1.6	WL 14.1 ± 0.6 + 3.2 ± 1.0 + 29.4%	LS 10.3 ± 0.5	NH 16.2 ± 1.1 + 5.8 ± 1.17 + 55.8%	WW 10.8 ± 0.4	WW 66 ± 3.7 + 35 ± 3.99 + 112.9%	WW 31 ± 1.5	LS 43 ± 3.9 + 16 ± 4.25 + 59.3%	LS 27 ± 1.7	WR 22 ± 1.4 − 1 − 4.4%	WW 22 ± 0.6
NH 30 ± 2.6 + 12 ± 2.67 + 66.7%	NH 18 ± 0.6	NH 21 ± 1.5 + 0 + 0.0%	LS 20 ± 1.1	NH 11.8 ± 0.4 + 2.5 ± 0.57 + 26.9%	NH 9.3 ± 0.4	LS 14.7 ± 0.7 + 3.6 ± 0.81 + 32.4%	WR 10.6 ± 1.2	NH 60 ± 3.8 + 24 ± 4.61 + 66.7%	WR 28 ± 2.2	WW 40 ± 2.1 + 16 ± 2.90 + 66.7%	WW 24 ± 2.0	NH 20 ± 1.3 + 2 + 11.1%	NH 18 ± 0.9
WR 24 ± 2.2 + 8 ± 2.34 + 50.0%	WR 16 ± 0.8	WR 14 ± 0.8 + 0 + 0.0%	WR 14 ± 0.6	WR 11.4 ± 0.8 + 2.5 ± 1.0 + 28.1%	WR 8.9 ± 0.6	WR 12.1 ± 0.6 + 1.5 ± 1.34 + 14.2%	NH 10.4 ± 0.4	LS 58 ± 3.3 + 30 ± 3.71 + 107.1%	LS 28 ± 1.7	WR 38 ± 3.4 + 14 ± 3.80 + 58.3%	WR 24 ± 1.7	LS 18 ± 1.3 + 1 + 5.9%	LS 17 ± 1.3

Note: The number of chicks contributing to the data in each cell of this table varies from 20 to 35 with an average of 27.

TABLE 3

ORDER OF RANK WITH RESPECT TO DEGREE OF RESPONSE

(a) Based on absolute increase over controls; (b) based on per cent. increase over controls

Rank	Comb				Thyroid				Gonad			
	♂ ♂		♀ ♀		♂ ♂		♀ ♀		♂ ♂		♀ ♀	
	a	b	a	b	a	b	a	b	a	b	a	b
1.	W.L.	N.H.	W.L.	W.L.	W.W.	W.W.	W.W.	W.W.	W.R.	W.R.	N.H.	N.H.
2.	N.H.	W.L.	L.S.*	L.S.	L.S.	L.S.	N.H.	N.H.	W.W.	W.W.	W.W.*	W.W.
3.	W.R.	W.R.	W.W.*	W.W.	W.L.	W.L.	W.L.	W.L.	W.L.	L.S.	L.S.*	L.S.
4.	W.W.	L.S.	N.H.	N.H.*	W.R.*	W.R.	L.S.	L.S.	L.S.	W.L.	W.L.*	W.R.
5.	L.S.	W.W.	W.R.	W.R.*	N.H.*	N.H.	W.R.	W.R.	N.H.	N.H.	W.R.	W.L.

* Equal in rank within the column.

corrected weight of the organ which is the top figure in each cell is followed, in the case of injected birds, with the simple excess over controls and this in turn is followed by the per cent. excess over controls. In each column the breeds are arranged in descending order of organ size, which does not necessarily correspond with descending order of sensitivity. Table 3 shows the order of rank of the various breeds with respect to sensitivity for each of the organs studied. The breeds are ranked (a) on the basis of absolute increase over controls and (b) according to per cent. increase over controls.

In Table 4 both the absolute and percentage differences in response between the various breeds are listed for each organ and each sex together with their standard errors. Because of the fact that each of these differences is based on four independent populations, *viz.*, an injected and a control group in each of the two breeds being compared, the standard errors are in many cases comparatively large. An asterisk means that the "t" value is between the .05 and .01 error points, while a dagger means that the odds on significance is greater than 99 : 1.

Fig. 1 shows the corrected organ weights of the control and injected birds for each breed and each organ in each sex. It provides a bird's-eye view of the results and enables one to make a rapid comparison of the relative weights of the different organs in the different breeds for both injected and control birds. Fig. 2 illustrates the relative degree of response in the different breeds. It provides a picture of how the breeds compare in sensitivity in a clearer manner than does Fig. 1. It shows the absolute increase in organ weight immediately followed by the per cent. increase, each being drawn to its respective scale.

COMB

As was expected, the large-combed sexually precocious Leghorn exceeded all others in its absolute comb increase. In three of the four cases (see Table 4) the odds on sig-

TABLE 4

DIFFERENCES IN THE DEGREE OF RESPONSE BETWEEN ALL POSSIBLE BREED PAIRS FOR EACH ORGAN EXCEPT OVIDUCT. THESE DIFFERENCES ARE SHOWN IN BOTH ABSOLUTE AND RELATIVE TERMS. THE BREEDS LISTED ON THE TOP EXCEED THOSE IN THE MARGIN BY THE AMOUNTS SHOWN IN EACH CASE

	Absolute difference					Percentage difference			
	W.L.	W.W.	L.S.	N.H.		W.L.	W.W.	L.S.	N.H.
W.W.	† 18 ± 5.6	.....	.....	.....		* 40.6 ± 18.9	.....	.....	.....
L.S.	† 19 ± 5.9	1 ± 3.5	.....	.....	♂♂	17.0 ± 21.9	− 23.6 ± 15.4	.....	.....
N.H.	* 13 ± 5.8	− 5 ± 3.4	− 6 ± 3.9	.....	Combs	− 4.2 ± 23.2	† − 44.8 ± 17.2	− 21.2 ± 20.5	.....
W.R.	† 17 ± 5.7	− 1 ± 3.1	− 2 ± 3.6	4 ± 3.6		12.5 ± 23.3	− 21.8 ± 17.4	− 4.5 ± 20.6	16.7 ± 22.0
W.W.	* − 3.8 ± 1.7	.....	.....	.....		* − 36.0 ± 17.5	.....	.....	.....
L.S.	− 0.7 ± 1.2	* 3.1 ± 1.6	.....	.....	♂♂	− 8.5 ± 14.1	27.5 ± 16.3	.....	.....
N.H.	0.7 ± 1.1	† 4.5 ± 1.5	1.4 ± 3.1	.....	Thyroids	2.5 ± 13.2	* 38.5 ± 15.5	11.0 ± 11.5	.....
W.R.	0.7 ± 1.0	† 4.5 ± 1.7	1.4 ± 1.3	0.0		1.3 ± 16.6	* 37.3 ± 18.5	9.8 ± 15.3	1.2 ± 14.5
W.W.	* − 3.9 ± 1.7	.....	.....	.....		† − 40.7 ± 17.5	.....	.....	.....
L.S.	1.0 ± 1.3	* 4.9 ± 1.6	.....	.....	♀♀	5.6 ± 13.5	† 46.3 ± 16.3	.....	.....
N.H.	− 1.2 ± 1.5	2.7 ± 1.8	− 2.2 ± 1.4	.....	Thyroids	− 17.8 ± 13.2	22.9 ± 18.3	− 23.4 ± 14.5	.....
W.R.	3.1 ± 1.7	† 7.0 ± 1.9	2.1 ± 1.6	* 4.3 ± 1.8		23.8 ± 17.9	† 64.5 ± 19.7	18.2 ± 16.2	* 41.6 ± 18.6
W.W.	− 3 ± 5.1	.....	.....	.....		− 28.7 ± 18.6	.....	.....	.....
L.S.	2 ± 4.9	5 ± 5.5	.....	.....	♂♂	− 22.9 ± 19.9	5.8 ± 23.4	.....	.....
N.H.	8 ± 5.6	11 ± 6.1	6.0 ± 5.9	.....	Gonads	17.5 ± 18.8	* 46.2 ± 22.5	40.4 ± 23.5	.....
W.R.	− 10 ± 6.5	− 7 ± 6.9	− 12 ± 6.8	† − 18 ± 7.3		* − 65.8 ± 28.8	− 37.1 ± 31.3	− 42.9 ± 32.1	† − 83.3 ± 31.4
W.W.	0.0	.....	.....	.....		− 19.6 ± 19.2	.....	.....	.....
L.S.	0.0	0.0	.....	.....	♀♀	− 12.2 ± 20.3	7.4 ± 24.1	.....	.....
N.H.	− 6.0 ± 4.4	− 6.0 ± 4.3	− 6.0 ± 5.3	.....	Gonads	− 23.9 ± 16.1	− 4.3 ± 20.7	− 11.7 ± 21.7	.....
W.R.	2 ± 4.9	2 ± 4.8	2 ± 5.7	8 ± 5.0		− 11.2 ± 20.7	8.4 ± 24.4	1.0 ± 25.2	12.7 ± 22.0

* Odds on significance > 19 : 1 ; † Odds > 99 : 1

nificance exceed 99:1. However, so far as percentage increases are concerned the Leghorn excess reaches the point of statistical significance only when compared with the low-ranking White Wyandotte. The most surprising thing in connection with the comb is the very large percentage increase in the New Hampshires, which was 67 per cent. as compared to 63 for the Leghorns. The odds on significance of the difference between New Hampshire and White Wyandotte exceeds 99:1. The White Rocks and Light Sussex with gains of 8 mg (50 per cent.) and 6 mg (45 per cent.), respectively, followed the Leghorns and New Hampshires but exceeded the White Wyandotte which showed a gain of 7 mg or only 22 per cent.

By comparison the females showed little stimulation. The Leghorns showed a gain of 4 mg or 14 per cent.; the Light Sussex, 2 mg (10 per cent.); the Wyandottes, 2 mg (8 per cent.) while the New Hampshires and White Rocks were not affected by the injections. In connection with the combs it is interesting to note that, in both sexes, the Leghorn has the largest natural comb weight, followed by the Wyandotte, with the White Rock bringing up the rear. In males, the Light Sussex and New Hampshires take third and fourth place, respectively, while in the females these two breeds are about equal.

THYROID

None of the basic breed differences in the thyroid weights of the control groups of either sex were statistically significant, but the injected chicks showed distinct breed differences in thyroid response. The Wyandotte is by far the most sensitive. Both sexes of the breed surpassed the other four in both absolute and relative thyroid response and in all cases except that of New Hampshire females the differences are greater than required for odds of 19:1. Gains of 65 per cent. and 79 per cent. in the Wyandotte males and females, respectively, were recorded as compared to 38 per cent. for

Light Sussex males and 56 per cent. for New Hampshire females which were next in order of gain in their respective sex groups. The Light Sussex males, however, are not significantly higher in response than any of the remaining three breeds, but the New Hampshire females do exceed the significant point both absolutely and relatively when compared with the low-ranking White Rock females. White Leghorns, which are generally used in thyrotropic assays, were third on the thyroid sensitivity list with White Rocks at the bottom.

A cursory examination of the thyroid data indicated the existence of a sex difference in the size of that organ. This was confirmed on analysis. The pullet chicks of the control lots averaged 1 mg heavier in thyroid weight, when comparisons were made within breeds. The odds on significance tested by "Students" method is approximately 99 : 1. There is also a distinct though statistically non-significant tendency for the degree of thyroid response in female to exceed that in males.

Gonads

While White Rocks were among the least responsive to hormone injections in so far as the comb and thyroid tissues were concerned, the testes of this breed showed an inordinately large increase. Although normally possessing testes fully one third smaller than those of the Leghorns, the male gonads of the White Rock chicks showed a response of 150 per cent. This greatly exceeds the 84 per cent. response of the Leghorn testes. In fact, the White Leghorn rate of response was second lowest, surpassing only that of the New Hampshires. Table 4 shows that while the White Rock testes consistently exceeds all others in both absolute and relative response, this excess reaches the significant point only when compared with the New Hampshire (absolutely and relatively) and the White Leghorn (relatively). The White Wyandotte testes give the second highest response, *viz.*, 35 mg or 113 per cent. This response is significantly higher than the low-ranking New Hampshire.

The control groups of all breeds had smaller testes than the Leghorns. With the exception of the New Hampshire testes these differences were statistically significant. This same breed relationship is found in the ovaries.

Compared to the testes, response of the ovaries was, on the whole, a good deal lower. Here the New Hampshire pullet chicks showed the greatest degree of response when measured in both absolute and relative terms. The Leghorns, which normally possess the heaviest ovaries, failed to keep the lead under hormone stimulation. However, none of the breed differences were statistically significant.

OVIDUCTS

The oviducts were practically unaffected by the treatment, as can readily be seen by referring to Fig. 1. In only three of the five breeds do the treated birds exceed the controls. Apparently the ovaries were not stimulated to elaborate estrogen at least in effective amounts.

EFFECT OF FAST-FEATHERING GENE

Because of the appearance of fast-feathering individuals in the predominantly slow-feathering breeds, such as White Wyandottes and White Rocks, an opportunity presented itself to test for the existence of any relationship between this gene and the rate of response to hormone. The sex-linked gene for slow feathering (K) is dominant to the fast-feathering gene (k). Results of the analysis of the data showed that at least under the conditions of this experiment, these two alleles do not differentially affect the response threshold in any of the organs studied.

DISCUSSION

Two parallel series of results emerge from this study. First, there exist basic breed differences in the size of certain primary and secondary sex organs of baby chicks. Breneman (1941) found such differences in the organs of

White Leghorns and Rhode Island Reds. He suggests that such differences be taken into consideration when hormone experiments are based on chicks belonging to several breeds.

The second point concerns itself with what are apparently genetically controlled differences in the response of these organs to the A.P. extracts. This breed specificity in response often overshadows the normal standing of breeds in respect to the natural size of these organs. Breeds which possess naturally heavier organs do not necessarily exhibit correspondingly higher or lower degrees of response.

Perhaps the most striking case in this connection is that of White Wyandottes. The thyroids of this rose-combed breed apparently have the lowest response threshold of any of the five breeds studied. The fact that both sexes of this breed showed the highest sensitivity lends strong support to the thesis that this is due to a gene control over the somatic threshold of response to thyrotropic hormone.

The White Rock breed presents another example of this phenomenon. Largely because of the naturally small size of comb, thyroid and ovary, the weights of these organs in the hormone-treated groups of this breed were, relatively, the smallest of the five breeds tested. Moreover, the rate of response was also of a uniformly low order. The White Rock testis, on the other hand, although also the smallest of the control groups, showed an extremely high sensitivity with an increase of 42 mg or 150 per cent. These are considerably higher than corresponding values for the Leghorns and confirms the findings of Nalbandov and Casida (1940) in respect to the relative sensitivity of these two breeds.

The high level of response exhibited by the White Rock testis is all the more surprising in view of past experiences in this laboratory with a closely related variety, the Barred Rock. The latter proved to be less suitable than Leghorns for work on the gonadotropins because of

its high response threshold. It is difficult to account for such a distinct intra-breed response differential, particularly since the White Rock is known to have originated as a recessive white mutant from the Barred variety. Originally, then, the two varieties differed in only one gene, the White Rock lacking the chromogen-forming dominant gene C possessed by the Barred. More recently certain strains of White Rocks have been altered by introducing White Wyandotte and White Leghorn blood with the result that some families and strains are known to lack the barring gene while some possess dominant white plumage; they also, undoubtedly, differ with respect to other cryptomeres. All this complicates the picture, but it is still possible that the lower threshold value of the White Rock testis is due to the original single gene difference between the Barred and White varieties. The relatively high sensitivity of the White Wyandotte testis, another breed which lacks the C gene, lends weight to this possibility. Plans are under way in this laboratory to test the relationship between this gene and testis sensitivity.

The low threshold of response of the White Rock testes contrasts sharply with the relatively low degree of sensitivity of the male chick combs in this breed. At the same time, combs of the male Leghorn chicks responded at a high rate compared to their testis tissue. This may indicate the existence of purely local organ specific response thresholds, or it is possible that a mere increase in testis size is no criterion of the amount of endogenous androgen which it produces. It also seems likely that the genotype exercises a control over ultimate organ and body size. Thus, no amount of androgen will make the Barred Rock male comb as large as that of a normal White Leghorn male.

The existence of a sex difference in the weight of the thyroid gland presented some interest in light of the earlier report of Riddle (1929), who found no significant sex difference in the weight of this organ in pigeons and

doves. Juhn and Mitchell (1929) presented inconclusive evidence on this point in adult Brown Leghorns. Findings of the present investigation are in agreement with those of Aberle and Landauer (1935), who showed that female chicks of the White Leghorn and Frizzle breeds have larger thyroids than the males.

The results of this investigation suggest some practical considerations. The fact that there exists inherent breed specific degrees of organ response to hypophyseal hormones should cause investigators to exercise greater care in the selection of breeds and varieties of chickens for use in endocrine investigations. Additional data being accumulated at this laboratory indicate that the same principle of a genetically controlled response in chicks exists with regard to both estrogens and androgens. The judicious selection of breeds should greatly increase the efficiency of hormones when chicks are used as experimental animals. It is entirely possible of course that other breeds or varieties exist which will prove more efficient in this type of work than those found most responsive in the present study.

Another point which may prove of practical value is the possibility that breeds, and more especially families within breeds, which are most sensitive to gonadotropins may prove to be superior in reproductive performance. For example, the New Hampshire females in this study have the most responsive ovaries, and although the differences between this breed and the others do not reach the point of statistical significance, there is good evidence that the New Hampshire is superior in its ovarian response. Might not this breed, in respect to its primary egg genes (referred to in the introduction), be genetically superior to the others? The laying ability of the New Hampshire has many champions, and although there is no unanimity with respect to this it does appear that the eggs of this breed hatch better than most others. Any superiority possessed by a breed by virtue of its complement of primary egg genes would be, as explained previ-

ously, largely masked by the protective genes and the environment. It would be difficult, therefore, to prove any relationship between, for instance, ovarian response to hormones and reproductive performance. However, this is a point which should be borne in mind.

SUMMARY

Baby chicks of both sexes of five commercially popular breeds of fowl, *viz.*, White Leghorn, New Hampshire, Light Sussex, White Plymouth Rock and White Wyandotte, were compared in respect to the degree of response (increase in weight) shown by their gonads, thyroids and comb when injected with a total of 18 mg of concentrated anterior pituitary extract per chick spread over a ten-day injection period. Controls were run in each sex and breed group. Organ weights were corrected to a uniform body weight by means of regression.

Comparisons between the control groups showed that breed differences exist in the natural organ weights. After treatment the relative organ weights in the different breeds are often reversed due to a breed differential in sensitivity of response.

When the breeds are compared on the basis of either absolute or relative increase over their untreated controls, certain organs of certain breeds were outstanding in the degree of their response. Chief among these were: (1) the combs of White Leghorn males; (2) the thyroids of both sexes in Wyandottes; (3) the testes of White Wyandotte and especially White Rock males; (4) the ovaries of New Hampshire females, although this point can not be considered as fully established in this study.

Different strains of these breeds of course will not necessarily show the same relative response. However, this study shows that breeds differ in their response to pituitary hormones. These breed differences are considered to be due to a genic control over the somatic response threshold.

Some practical implications of these findings are discussed.

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REVIEWS AND COMMENTS

EDITED BY CARL L. HUBBS

In this section reviews and notices are given of current publications on general biology and of specialized works which have an important bearing in this general field. Emphasis is given to books and major articles which fall within the special scope of The American Naturalist, in that they deal with the factors of organic evolution.

Reviews and Comments are meant to include also such general discussions, reports, news items and announcements as may be of wide interest to students of evolution. Except as otherwise indicated, all items are prepared by the Section Editor, Dr. Carl L. Hubbs, University of Michigan, Ann Arbor, Michigan. All opinions are those of the reviewer.

Family Treasures. A Study of the Inheritance of Normal Characteristics in Man. By David L. Whitney. Lancaster, Pa.: The Jaques Cattell Press, "1942" [1943]: 1–299, figs. 1–234. $3.50.

This finely illustrated and interestingly written book was prepared "for amateurs in the study of human heredity and for those who are interested in personal inheritance of family traits. It is a simple presentation mainly by photographs of many of the normal traits appearing in families of two or more successive generations." It will therefore have a strong popular appeal, for the mind of man, when unfettered by scientific training, learns and generalizes from the sort of impressive examples that Whitney describes and figures. The "amateur" will seldom stop to query whether chance might explain familial resemblances in selected samples. He will be relieved at the almost total omission of pedigrees, correlations and ratios (the nearest approach to a statistical treatment lies in enumerating the results of the trial flippings of a coin and of the blind selection of pairs of marbles from a supply of black and white ones). Some critical folks may wonder whether such teaching by the precept of example may not diffuse a false conception of the scientific method. Disapproval will also come from an almost opposite quarter, from those social scientists who deny that heredity is significantly involved in determining the behavior, the temperament and the mental

abilities of the human individual. Without concealing his own naturalistic philosophy, Whitney compromises with the humanists in conservatively stating that human inheritance *seems* to follow the laws that operate in animals, and that man's ancestors were *probably* animals. He deals rather effectively and very amusingly with the common superstitions and myths concerning the role of providence and of the devil in human heredity, and with prenatal influences and the inheritance of acquired characters.

Levels of Integration in Biological and Social Systems. Edited by ROBERT REDFIELD. Biological Symposia, Vol. VIII. Lancaster, Pa.: The Jaques Cattell Press, 1942: i–v, 1–240, 7 figs. $2.50.

IN this significant contribution to the philosophy of biology and of sociology, ten scientists have presented their data and views on the social system and its biological precursors and analogs. An attempt to integrate their views is made by ROBERT REDFIELD.

The common thread in the contributions is the development on different biological levels of similar systems of integration, under which individuals become coordinated in larger units. The expanded units, it is repeatedly affirmed, possess attributes of the individual organism. The organismic concept is therefore stressed, particularly in Redfield's Introduction and in ALFRED E. EMERSON's chapter, Basic Comparisons of Human and Insect Societies. Essential features of the individual—life, origin, growth, survival in the struggle for existence, maturity, reproduction, and death—are indicated either by definite claims or by connotation to be characteristic also of the species and of the major phyletic lines; of integrated colonies; of ecological communities, and of various types of social groups. Parallels, as of mutual interdependence and division of labor, feature the integrated colony, including, as LIBBIE HYMAN reports, the metazoan individual; populations of lower pre-social animals, including

the infra-social insects, which are discussed from an ecological viewpoint by THOMAS PARK; the highly complex, heredity-based systems of insects; the social organization, dominated by rank-order, in birds and mammals, interestingly treated by W. C. ALLEE; the sub-human Societies of Monkeys and Apes—the subject of C. R. CARPENTER's contribution; the Societies of Primitive Man, presented by A. L. KROEBER; and Modern Society, by ROBERT E. PARK. Evolutionary advance in the successive categories of social systems is mentioned, even in an age when a cynic might be filled with doubts.

Distribution and Variation of the Horned Larks (Otocoris alpestris) of Western North America. By WILLIAM H. BEHLE. Univ. Calif. Publ. Zool., 46, 1942: 205–316, figs. 1–3. $1.25.

THE horned larks are shown to be highly variable. Their variation and differentiation is to a large degree individual, developmental and sexual, but is in part correlated with geography. Numerous geographical subspecies are therefore separable. These local forms occupy distinct faunal areas, life zones and physiographic areas. Subspeciation is tied in with environmental changes, as of climate, and is regarded as "a continual process of adjustment and adaptation in the organism, which has resulted in continued harmony, or approach to harmony, between the organism and its environment." Racial colors tend to correspond adaptively with soil colors, as they do in African larks. The breakdown of subspecies into minor local races and the close agreement between some geographically remote subspecies is perhaps to be explained on the basis of such adaptations in color. In other ways the local forms show adaptations to their particular habitats, and the horned larks as a group are strikingly adapted for life on open areas with a small amount of vegetational cover. Of the "ecological laws," Bergman's and Gloger's apply, but Allen's does not.

Behle breaks away in a restrained fashion from ornithological precedent. His treatment is more detailed than usual but is still in large part impressionistic. Correlations between plumage color and soil color are affirmed but not measured. Critical speciational relations, as in areas of subspecific intergradation, are extensively discussed, without the test that quantitative data would supply. Important advances are made toward the better understanding of speciation, but with statistical support these contributions would have been more securely established and more convincingly presented.

The Genus Nysius and Its Allies in the Hawaiian Islands (Hemiptera, Lygaeidae, Orsillini). By ROBERT LESLIE USINGER. Bishop Museum Bull. 173, 1942: i, 1–167, 9 text figs., 12 pls. $2.00.

In the reviewer's opinion, this is the finest piece of systematic work ever published concerning any group of Polynesian insects. How much easier would be the tasks of future workers if more authors would adopt a style similar to Usinger's!

The bug-tribe Orsillini is spread throughout the world, but its greatest proliferation is in Hawaii where there are 84 endemic forms contained in 5 genera (4 endemic). The closest approach to the Hawaiian fauna in comparative development and complexity is that of New Zealand (but the two faunas are not directly related).

"The peculiar Hawaiian orsilline fauna exceeds all the others in complexity and degree of divergence and may reasonably be considered the oldest." The reviewer does not believe that in insular faunas divergence and complexity necessarily indicate great age as compared to continental areas. Explosive speciation and great diversification are characteristics of islands known to be geologically young. The Hawaiian Orsillini have been derived from ancestral immigrants, therefore they must be younger than some other faunas.

"The endemic genera *Neseis, Oceanides* and *Glyptonysius* probably migrated down the long series of leeward islands before the main islands of the present day were built." This is good reasoning, and it finds support in other groups of organisms. "They must have arrived not later than earliest Tertiary times as judged by mainland evolutionary rates, or perhaps later than this considering that evolution has taken place in the absence of severe competition." If Usinger means that the ancestors of these forms arrived in the main Hawaiian islands at such an early date, I believe that he is overestimating the ages of the islands. His second conclusion appears by far to be the most likely; his first is not supported by geological facts.

Usinger's conclusions are Neo-Darwinian. He finds gradations from widespread, variable species to scarcely differentiated forms to polytypic species to supra-species to geographical subgenera. One current school might say that Usinger is dealing with micro-evolutionary segregates and not "good species." However, a review of the text and a glance at the superb illustrations (which convey only a small part of the numerous differences of the living animals) should eliminate such thought. "The conclusion seems inevitable that geographical isolation or host isolation or both may be sufficient to set in operation the processes of species formation, while the biotic environment plays an all-important role in determining the rate and limits of this evolution. A disharmonic insular area with great gaps in its environment allows many non-lethal mutations to persist, whereas a fiercely competitive mainland environment rigidly rejects all but the best adapted, thus favoring adaptive evolution by natural selection." Such conclusions can hardly be escaped by open-minded students of mid-Pacific biotas.

Usinger wisely discards the worn-out opinion of those who believe the Hawaiian islands to be remnants of a drowned Pacific continent and logically adopts the "stepping stone" hypothesis and considers that migration has

taken place by short jumps from island to island along island chains. He also logically includes the decadent leeward islands as being of great importance as a migration lane which, by a circuitous route, leads to the Australian, Papuan and Oriental regions. This accounts "for the complete absence of many groups, such as the Orsillini, from southeastern Polynesia, the very islands where they would be expected to occur had the fauna of Hawaii been derived directly from the southwest." The relationships of the group show a derivation from the west and southwest Pacific. "An interesting anomaly is the lack of relationship with Micronesia and American species." Wind is considered to have been the principal agent in the dispersal of these small bugs.

Usinger believes that "To the evolutionist they represent the first case of tremendous proliferation of species in insular areas which presents a possibility of experimental analysis." The reviewer agrees, because a sound, adequate foundation is available; the habits, hosts and life histories of several species are known; there is every category from local varieties to genera to work with; and at least some of them are easily raised in captivity. We hope that the author may some day have an opportunity to carry on further research on this problem in Hawaii where it may lead to some most significant evolutionary facts.

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NOTICES OF NEW BOOKS

Papers from Tortugas Laboratory, Volume XXXIII. Carn. Inst. Wash. Publ. 524, 1942: i–iii, 1–195, pls. 1–7, 73 figs. $1.50 (paper), $2.00 (cloth).—It is gratifying to see that the Papers from Tortugas Laboratory are still appearing. The thirty-third volume is made up of six contributions. HAROLD W. MANTER describes gasterostome trematodes from Tortugas. LEONARD B. CLARK and WALTER N. HESS describe in detail the swarming of the Atlantic palolo worm, concluding that "although reproduction occurs over a much longer period than was previously held,

it is thought that the lunar cycle, maturity of the palolo worm, and wave action are the three main factors determining the time of a major swarm." The same authors treat the responses of this worm to light, and correlate its light reactions with the uniform time of night at which it swarms. RALPH WICHTERMAN reports on the structure and division of three new ciliates from the littoral earthworm of Tortugas, and describes another new ciliate, representing a new family, and its symbiotic zooxanthellae. JOHN H. DAVIS, JR., concludes the volume with a long and interesting account of the vegetation and topography of the Sand Keys of Florida.

The Embryology of Eleutherodactylus nubicola, an Anuran Which Has No Tadpole Stage. By W. GARDNER LYNN. Carn. Inst. Wash. Publ. 541, 1942: 27–62, pls. 1–5, figs. 1–40.—This Jamaican leptodactylid deposits large unpigmented eggs on land. After a developmental period of about 26 days they hatch as completely formed frogs. The numerous developmental modifications include absence of external and internal gills, open gill slits, specialized larval mouthparts, coiled intestine, larval adhesive organs, and true operculum. Development of the chondrocranium and jaws is characterized by lack of the larval specializations normally occurring in leptodactylid tadpoles. Supra- and infrarostral cartilages are not differentiated, Meckel's cartilage is elongate, and the form and articular relations of the quadrate are essentially as in the adult. The larval quadratocranial commissure and processus muscularis do not appear. Lynn points out the scattered occurrence of direct development in the Salientia, but suggests that the factors bringing it about may be the same in each case. "It is suggested that many of the features which characterize the abbreviated 'larval' history may be brought about as a result of precocious activity of the thyroid gland, and further investigation of this aspect of the matter is now being carried out."—GRACE L. ORTON.

Picture Book of Insects. By ALBRO T. GAUL. New York: Lothrop, Lee & Shepard Co., 1943: 1–40, illustr. $1.50.—Designed for eight to twelve year olds, this book comprises a series of attractive plates, accompanied by text statements that remind one of the well-written labels of a good museum exhibit. It is well designed to catch the eye and to inspire interest in insects.

Meet the Natives. An Easy Way to Recognize Rocky Mountain Wildflowers, Trees and Shrubs of the Central Rocky Mountain Region. By M. WALTER PESMAN. Denver, Colo. (372 S. Humboldt St.): Author's Edition, 1942: 1–216, many figs. $1.25. —"Meet the Natives" is, as the title implies, a pleasant and informal introduction to the wildflowers, trees and shrubs of the central Rocky Mountains. Instead of the usual type of keys employed in manuals, the plants are grouped first according to life zone, then arranged according to color of the flowers. To emphasize the color and make the book easier to use, colored paper corresponding to the flower color is used. The plants in the various sections are arranged according to flowering time, starting with the early blooming plants. Common and scientific names are both given and are followed by a brief characterization of the species. Water plants, weeds and vines are listed separately to facilitate identification. Many excellent photographs and line drawings are included. The drawings in particular emphasize the diagnostic features of the plants. For those who are interested primarily in the names of plants and the places they grow, this is an excellent introduction to the study. Young people whose interest in the out-of-doors is just being awakened will find in this book a charming and not at all formidable way to learn the plants. It should be a welcome addition to every camp and school library. The definitions of some frequently used scientific terms and the bibliography will be useful to those who are stimulated to further study. The small size and attractive appearance will appeal to the hiker and nature lover. The professional botanist will find it of interest primarily because of the photographs and drawings.—Mrs. HELEN V. SMITH.

SHORTER ARTICLES AND DISCUSSION

EVIDENCES OF CANNIBALISM IN THE TADPOLES OF THE FROG RANA PIPIENS

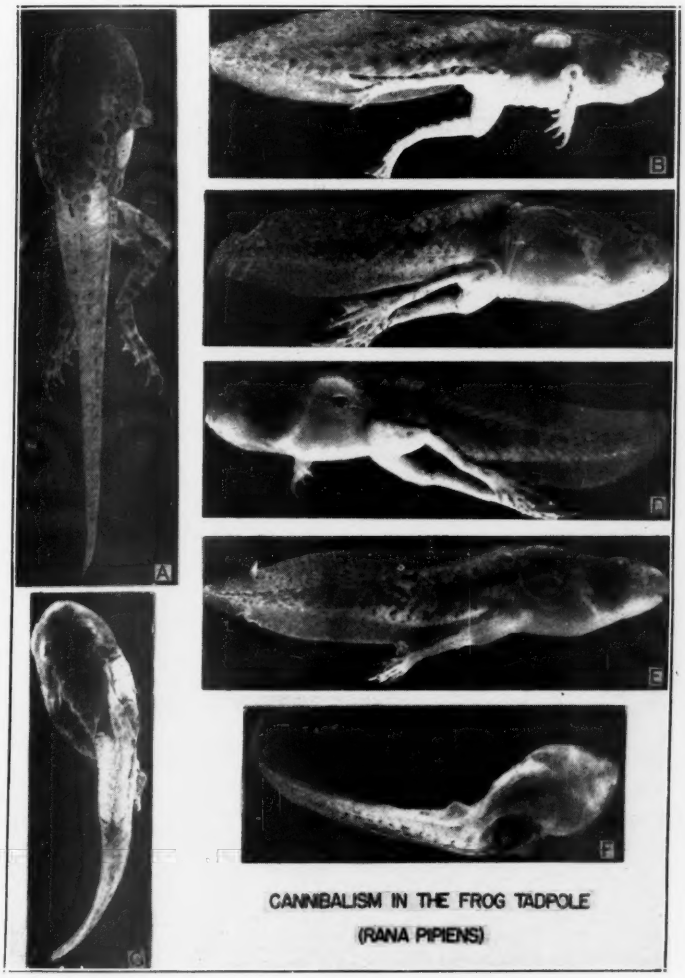
LARVAL salamanders frequently snap off each other's gills, legs or tails when hungry and crowded. According to Noble (1931) some terrestrial salamanders will leave part of their tail in the hand which attempts to seize them, a protective process somewhat similar to the autotomy of the claw of the lobster. The normal diet of frogs and toads may be animal or plant food, or both, depending partly upon the species and the habitat. Generally the larvae are vegetarian until metamorphosis, when the bulk of the food changes to Insects, Annelids and Arthropods. Many of the larger frogs (*i.e.*, bullfrogs) are cannibalistic in that they will eat smaller frogs such as the green or the leopard frogs. The tadpoles of *Ceratophrys ornata* are largely cannibalistic (Noble, 1931) but generally feed on the larvae of other frogs. Among the tadpoles there seems to be a definite food preference; in some it is exclusively vegetarian. Since all amphibia are voracious eaters whether as larvae or as adults, a sparcity of food accentuates the otherwise incidental cannibalistic tendencies of various forms.

The ability to regenerate is not related to the liability to injury. Newts may regenerate their hyoids (Bogoljubsky, 1924), frogs may regenerate their lungs (Westphal, 1925), and the exposed gills of the axolotl may be readily regenerated although not always in the same form (Wurmbach, 1926). The power of regeneration is gradually reduced in phylogeny (Korschelt, 1927). Larval regeneration is more frequent and more perfect than that of the adult amphibian, due in part to the forming of blastemas of more or less undifferentiated tissue which is therefore labile. Ubisch (1923) found that regeneration was in general better the more posterior the site on the larva.

Cannibalism among *Rana pipiens* tadpoles has been observed only in cultures where there are dead tadpoles that are devoured, along with other materials on the bottom of the containers. This paper presents evidence of true cannibalism among living tadpoles of this species.

Rana pipiens tadpoles were kept in groups of 100 in tanks measuring 24″ x 12″ x 6″ under controlled conditions of light and

PLATE I



FIGS. A to D show various degrees of injury due to cannibalism in Rana pipiens. Regeneration of these areas is achieved by isolating the tadpoles in fresh water. FIGS. E to G show viscera protruding from the injured area, after cannibalistic attack. Such injuries rarely heal over, even under the best of conditions.

(Note: Same general position of all cannibalistic attacks.)

temperature. They were fed maximally on spinach which had been thoroughly washed (to remove insecticides) and had been partially boiled (Rugh, 1941). The amount of cannibalism in the various tanks was not constant, but it occurred even under the most favorable conditions. The tadpoles were seen to nip each other quite readily, the chief sites being the head, the tail and the body wall just anterior to the junction of the tail. The head integument is relatively tough, while the tail is generally active, so that these two regions were the least vulnerable. The lesions were most frequently found postero-laterally on the body wall where the integument is rather thin and through which may be seen the highly colored liver and other viscera. They were almost invariably oval or rounded. The first appearance of the lesion is brown, due to the presence of underlying melanophores. Necrosis of the epidermis soon occurs, resulting in a white patch which is completely avascular. At this stage the melanophores and the xantholeucophores underlying the necrotic epidermis appear perfectly normal. The vascular pattern is quite normal except for a slight hyperaemia. Frequently the body wall is further perforated by continued nipping, resulting in death to the tadpole within six to twenty-four hours. When perforation of the muscular body wall is achieved and the viscera are visible, recovery is impossible.

The above conditions might well have escaped observation due to the rapid regenerative powers of the larval integument except for the fact that some of the tadpoles were subjected to an experimental environment in connection with a series of experiments on diet. To the spinach suspension was added $M/10^{-6}$ solution of methyl cyanide. This cyanide prevented proper regeneration of the integument, hence they accentuated the appearance of the lesions and made these observations possible. When such animals were returned to pure spring water and somewhat segregated, the lesions generally healed rather quickly. It has been conclusively demonstrated that the methyl cyanide in no way caused the lesions but once the integument was broken (by cannibalism or otherwise) this dilute cyanide solution prevented the normal regeneration of the tadpole integument.

The approximate size of the tadpole when cannibalism begins is about 65 mm in total length, 22 mm in body length, and 6 mm in hind-limb length. In an experimental group, poorly nourished on cabbage and treated with methyl cyanide, no lesions were seen.

This is explained on the assumption that cannibalism bears a relationship to the size of the tadpole and these starved tadpoles were relatively very small. Also, small larvae are able to move quickly and avert attack.

In experimental work with amphibian larvae it is of utmost importance that they be fed adequately and that they be not crowded, two conditions which, if not observed, will increase the probability of cannibalism.

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THE HISTOLOGICAL BASIS OF A SPECIFIC DIFFERENCE IN LEAF TEXTURE

IN a previous study of the species problem in *Uvularia* (Anderson and Whitaker, 1934), a working hypothesis was advanced to account for certain general differences between *U. perfoliata* and *U. grandiflora.* Since *Uvularia perfoliata* in flower, leaf and inflorescence is characteristically smaller and more graceful than *Uvularia grandiflora* (which by comparison seems coarse and irregular) it was suggested that this difference had its basis in a fundamental nuclear difference between the two species. Such a difference would be expressed, more or less similarly, in various parts of the plant. On this explanation the neater and more finished leaf texture of *U. perfoliata* would depend upon

the general tendency of that species to produce smaller, more regular cells.

When a few preliminary trials showed that the above hypothesis could be readily tested by the celloidin peel technique (Long and Clements, 1934), specimens of each species were selected from the herbarium of the Missouri Botanical Garden. Celloidin peels were made from the upper epidermis of a corresponding leaf from each plant. The outlines of the epidermal cells were then drawn with a camera lucida.

The results confirmed the hypothesis. The epidermal cells of *Uvularia grandiflora* tend to be larger, more irregular and more variable than those of *U. perfoliata*. The gross effect of this tendency to larger, more variable, more irregular cells is a tendency to a coarser, cruder leaf-texture.

SUMMARY

The differences in leaf texture between *U. grandiflora* and *U. perfoliata* depend on cellular differences. The larger, more irregular, more variable epidermal cells of *U. grandiflora* give it a coarser, cruder texture than that of *U. perfoliata*.

CONCLUSIONS

The results reported above provide concrete data on an aspect of the species problem which many naturalists have felt but few have discussed. Closely related species commonly differ from one another not only by a few trivial (albeit taxonomically useful) details but also by a host of vague tendencies variously expressed throughout the organism (Anderson and Whitaker, 1934; Anderson and Ownbey, 1939). This second sort of difference, however useless it may be to the taxonomist and however mystical it may appear to those without biological insight, rests upon a firm foundation theoretically. Species are now known to differ by their nuclei. Consequently in considering any two species one sees the same set of differences more or less harmoniously expressed throughout the organism. The same intracellular influences which produce a tendency towards a certain type of leaf produce correlated changes in the inflorescence. Though difficult to apprehend and even more difficult to put into words such tenuous correlated differences in texture and aspect come closer to the very essence of the species problem than do discrete, readily definable, characters. In his discussion of funda-

mental leaf form Velanosky (1905) said: "Die Blattform entspricht nicht nur den biologischen Zwecken und morphologischen sowie historischen Ursachen der beterffenden Pflanze, sondern passt sich auch dem ganzen Baue und Stil der Pflanze harmonisch an, wodurch die Pflanzen nicht selten ein prachtvolles Exterieur gewinnen, welchem strenge, äesthetische Regeln zugrunde liegen." One might adopt his generalization to the species problem and say that species differences are harmoniously expressed through the whole architecture of the plant, through the operation of basic esthetic laws.

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WHITE-EYED MUTATION IN PHORMIA REGINA MEIGEN

SEVERAL white-eyed individuals of *Phormia regina* Meigen have been obtained in March, 1942, through the courtesy of the Department of Animal Behavior of the American Museum of Natural History, New York. The appearance of the white-eyed mutants was noticed previously in the mass cultures kept by the Department. A wild-type strain, with reddish-brown eyes, has been established at the laboratory of the Bronx High School of Science from a single female caught in New York City. The flies were bred at room temperature, 70° to 72° F., sheltered from direct sunlight; the development from egg to adult takes about three weeks. Cane sugar, egg albumen and water were given to the flies as food, and pieces of liver or lung were introduced into the container on alternate days, for egg-laying. A true breeding

white-eyed strain was established without difficulty. The eye color in freshly hatched flies is pure white, but with age it acquires a yellowish or pinkish tinge.

In the first two experiments white-eyed females were crossed with red-eyed males, and red-eyed females with white-eyed males, respectively. The F_1 generation consisted of red-eyed flies of both sexes. Direct F_2 progenies were obtained; they included flies with red and with white eyes. In the third experiment F_1 females were back-crossed with white-eyed males; white and red-eyed flies appeared in the progeny.

The numerical relations are shown in Table I.

TABLE I

		First Experiment		Second Experiment		Third Experiment	
		Red ♂	White ♀	Red ♀	White ♂	Red ♀	White ♂
Females		482	169	732	247	108	120
Males		475	159	761	243	130	112
Total	Obs.	957	328	1493	490	238	232
	Exp.	964 ± 15.5 | 321 ± 15.5		1487 ± 19.3 | 496 ± 19.3		235 ± 10.8 | 235 ± 10.8	

It is clear that the white-eyed condition is due to a recessive autosomal gene. In this respect the mutant in *Phormia regina* Meigen behaves like similar mutants in *Psychoda alternata* Say (Turner, 1923) and *Lucilia cuprina* Wied (Mackerras, 1933). White-eyed mutants are also known in several species of Drosophila, but in all these the mutant is sex-linked. Provided that the white-eyed mutants in Phormia, Lucilia and Drosophila are homologous, we have here either a case of translocation between the X-chromosome and one of the autosomes or a transfer of the sex-determining factors from chromosome to chromosome in the phylogeny.

I am grateful to Professor T. Dobzhansky, of Columbia University, for his guidance in connection with this experiment.

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